Molekulare Untersuchungen zur Spezies-spezifischen Toxizität entwicklungsneurotoxischer Substanzen

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1. Einleitung

1.1. Entwicklungsneurotoxizität

Die Identifizierung von Chemikalien mit entwicklungsneurotoxischem Potential gewinnt zunehmend wissenschaftlich sowie gesellschaftspolitisch an Bedeutung (Goldman and Koduru 2000). Entwicklungsneurotoxizität beschreibt die funktionellen und morphologischen Effekte exogener Noxen auf das sich entwickelnde Nervensystem, die durch prä- und postnatale Exposition zu pathologischen Gehirnveränderungen führen. Derartige Effekte äußern sich bereits bei Kleinkindern in neurologischen Defiziten wie einem verminderten Intelligenzquotienten (IQ), Lernschwäche oder Aufmerksamkeitsdefizitstörung. Resultierend daraus entstehen im Allgemeinen hohe Kosten für individuelle Förderungen und Medikationen sowie Kosten für die Gesellschaft, die durch eine verminderte individuelle Leistungsfähigkeit hervorgerufen werden. Diese sind nicht zu unterschätzen, da beispielsweise in den USA etwa 17 % aller Kinder unter 18 Jahren neurologische Defizite aufweisen, die zum einen auf genetische Prädisposition und soziale Umstände zurückzuführen sind, zum anderen jedoch auch mit der Exposition gegenüber entwicklungsneurotoxischen Substanzen in Verbindung gebracht werden können (Schettler 2001).

Des Weiteren manifestieren sich Effekte auf das fetale Gehirn über das ganze Leben und beeinträchtigen damit nicht nur das betroffene Individuum und dessen Familie, sondern auch die Gesellschaft und die sozialökonomische Entwicklung, wie in Abbildung 1-1 dargestellt ist.

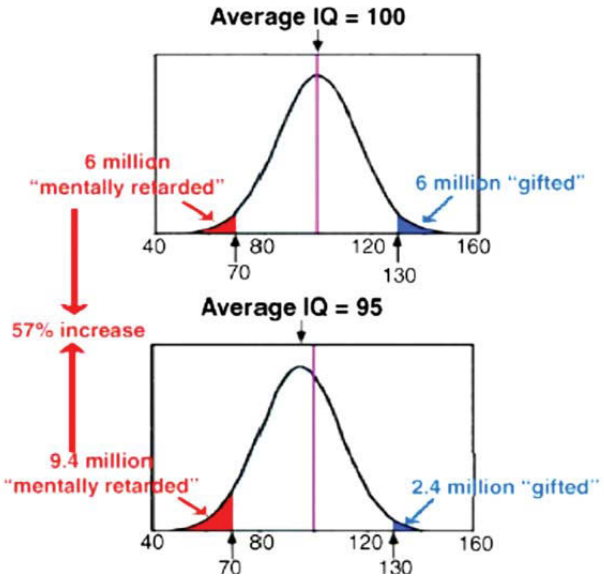

Abbildung 1-1: Sozialökonomische Auswirkungen einer Verminderung des mittleren Bevölkerungsintelligenzquotienten um 5 Punkte (Mt. Sinai Children's Environmental Health Centre).
Eine Verminderung des mittleren Bevölkerungsintelligenzquotienten um nur 5 Punkte führt dazu, dass der Anteil an mental Retardierten in der Bevölkerung um 57 % ansteigt.

Die Tatsache, dass verschiedenste Chemikalien die Gehirnentwicklung stören können, liegt vor allem darin begründet, dass das sich entwickelnde zentrale Nervensystem besonders empfindlich gegenüber der Schädigung durch Toxine ist (Rice and Barone Jr 2000; Rodier 1995). Die Gehirnentwicklung ist durch komplexe biologische Prozesse, die strengen Kontrollmechanismen unterliegen und eine lange Zeitspanne überdauern, gekennzeichnet. Zu diesen Prozessen zählen zellzahlregulierende Vorgänge wie Proliferation und Apoptose neuraler Progenitorzellen sowie die kontrollierte Differenzierung solcher Zellen zu reiferen neuralen Effektorzellen, welche vom Ort ihrer Entstehung zu ihrem Bestimmungsort gelangen müssen. Deshalb ist neben Proliferation, Apoptose und Differenzierung auch die Migration essentiell für die Ausbildung einer funktionellen Hirnarchitektur. Für die Reifung neuronaler Zellen sind Synaptogenese, die Ausbildung von Axonen und Dendriten und auch die Myelinisierung von großer Bedeutung. Die daraus resultierende Bildung neuronaler Verbindungen und Netzwerke ist dabei essentiell für den Aufbau einer funktionellen elektrophysiologischen Umgebung (Andersen 2003). Folgende Abbildung 1-2 stellt die Komplexität sowie den zeitlichen Ablauf der während der Gehirnentwicklung ablaufenden Prozesse dar.

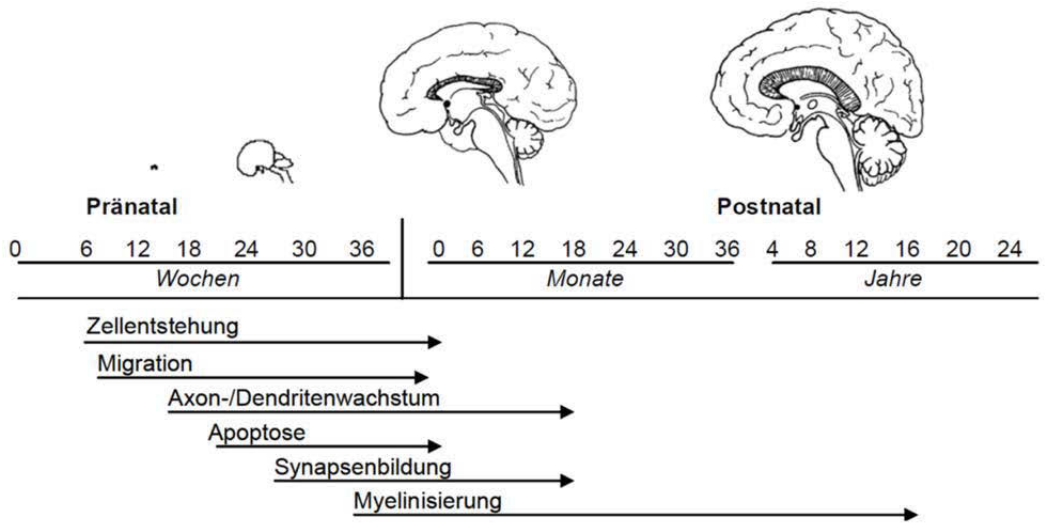

Abbildung 1-2: Schematische Darstellung der Gehirnentwicklung (nach Andersen 2003).
Die essenziellen Vorgänge der menschlichen Gehirnentwicklung sind in Abhängigkeit der prä- und postnatalen Entwicklung dargestellt.

Die einzelnen Prozesse der Gehirnentwicklung können mehrere Wochen, Monate und sogar Jahre überdauern und müssen zeitlich streng koordiniert erfolgen (Abbildung 1-2). Damit ergeben sich spezifische kritische Perioden, in denen unterschiedliche Noxen durch verschiedene Mechanismen die Entwicklung des Gehirnes stören können. Die lange Zeitspanne der Gehirnentwicklung determiniert also die Empfindlichkeit dieses Organs nicht nur während der embryonalen und fetalen Entwicklung, sondern auch postnatal bis hin zur Pubertät. Die

pränatale Sensitivität gegenüber toxischen Chemikalien wird außerdem durch eine noch nicht vollständig ausgebildete Blut-Hirn-Schranke verstärkt. Dadurch können neben lipophilen auch hydrophile Noxen und sogar Ionen in das fetale Gehirn gelangen und dort die Entwicklung stören (Claudio et al. 2000). Weiterhin sind fremdstoffmetabolisierende Enzymsysteme sowie die Exkretion exogener Substanzen im Fetus nur rudimentär ausgeprägt, wodurch die Sensitivität weiter gesteigert wird (Bondy and Campbell 2005).

In der Vergangenheit haben schwerwiegende Vergiftungsfälle mit beispielsweise Methylquecksilber oder polychlorierten Biphenylen eindrucksvoll gezeigt, dass Chemikalien mit den menschlichen Hirnentwicklungsprozessen interferieren können, was sich später in Neuroentwicklungsstörungen der Kinder äußern kann (Grandjean and Landrigan 2006). Aus diesem Grunde sind verschiedene Interessensgruppen (Regulatoren, akademische Wissenschaftler, Industrievertreter) auf beiden Seiten des Atlantiks zu der Übereinkunft gekommen, dass eine Evaluierung des entwicklungsneurotoxischen Potentials von Chemikalien unumgänglich für eine umfassende Risikobewertung für die menschliche Gesundheit ist (Bal-Price et al. 2015a; Crofton et al. 2011). In diesem Zusammenhang wurde die ISTNET (International STakeholder NETwork) Initiative ins Leben gerufen, um den Bedarf der regulatorischen Ebene mit der akademischen und industriellen Forschung für Entwicklungsneurotoxizitätstestung in Einklang zu bringen.

1.2. Sicherheitstoxikologische Testung auf Entwicklungsneurotoxizität

Zur Untersuchung von Entwicklungsneurotoxizität sind derzeit noch Tierversuchsstudien in der Ratte im Rahmen der Risikoabschätzung von Chemikalien verpflichtend. Dies gilt in Europa für Substanzen, welche neurotoxisches oder endokrin disruptierendes Potential aufweisen, während in den USA zudem alle Pestizide auf Entwicklungsneurotoxizität getestet werden müssen. In diesem Zusammenhang stehen Richtlinien der OECD (Testing Guideline 426) sowie der U.S. EPA (OPPTS 870.6300) zur Verfügung (OECD 2007; USEPA 1998), die aufwendige morphologische Gehirnuntersuchungen sowie Verhaltenstests und die Untersuchung von Biomarkergenen für Gliosen und Zytotoxizität vorsehen. Neben hoher entstehender Kosten und großem Zeitbedarf (Crofton et al. 2012) sind dabei vor allem die enormen Tierzahlen problematisch. So werden für die Durchführung solcher Tierversuchsstudien 140 Muttertiere und 1000 Jungtiere pro Substanztestung benötigt. Gerade im Zusammenhang mit der REACH-Verordnung, die die Testung von etwa 30.000 Altstoffen hinsichtlich ihres toxischen Potentials vorschreibt, und auch im Einklang mit den drei R's (Reduce, Refine,

Replace) nach Russell und Burch (Russell et al. 1959) sind solche Tierversuche vor allem aus Gründen des Tierschutzes nicht mehr tragbar.
Weiterhin bergen Speziesunterschiede zwischen Ratte und Mensch das Problem der Extrapolation experimentell ermittelter Grenzwerte auf den Menschen (Coecke et al. 2007; Lein et al. 2007; Lein et al. 2005). So ist die geringe Prädiktivität der Ergebnisse aus Tierstudien für den Menschen bereits aus der Pharmakologie bekannt (Leist and Hartung 2013). Eine Studie von Seok und Mitarbeitern (2013) zeigte eindrucksvoll, dass in den Forschungsfeldern Entzündung, Sepsis und Infektion die Genantworten nach Verletzungsstimulus zwischen Mensch und Maus kaum korrelierten und somit von Effekten in der Maus nicht auf Effekte im Menschen geschlossen werden kann. Dies spiegelt sich auch in dem Versagen jedes einzelnen der über 100 therapeutischen Ansätze, die im Tiermodel gegen Sepsis entwickelt wurden, wider. Weitere prominente Beispiele bieten die Erfahrungen mit Thalidomid und TG1412, deren verheerende Effekte im Menschen nicht aus den vorhandenen Tierstudiendaten abgeleitet werden konnten (Stebbings et al. 2007). Des Weiteren können Nager auch das Gefährdungspotential von Pharmazeutika überschätzen (Basketter et al. 2012; Gold et al. 2005), sodass vielversprechende Kandidaten frühzeitig aus dem klinischen Entwicklungsprozess ausscheiden.

Daraus ergibt sich ein steigender Bedarf für die Entwicklung und Validierung neuer Ansätze für die Toxizitätstestung, mit Hilfe derer Chemikalien hinsichtlich ihres entwicklungsneurotoxischen Potentials zuverlässig getestet und gegebenenfalls für weiterführende Testungen priorisiert werden können (Crofton et al. 2011).

1.3. Paradigmenwechsel in der Toxikologie

Im Jahr 2007 hat das US National Research Council in dem Bericht „Toxicity Testing in the 21st Century: A Vision and a Strategy“ vorgeschlagen, dass neue Testmethoden und Modelle entwickelt werden sollen, mit denen zum einen die Kosten und der zeitliche Aufwand der toxikologischen Testung reduziert werden können und zum anderen eine schnelle Datenerfassung zur Risikobewertung sowie eine bessere Identifizierung toxikologischer Mechanismen möglich gemacht wird. Gleichzeitig soll die Vergleichbarkeit der Situation im Menschen mit den bisher verwendeten Tiermodellen stärker hinterfragt werden, wobei die Untersuchung von Speziesunterschieden in den Fokus gerückt und stärker berücksichtigt werden sollen (Crofton et al. 2012; Gibb 2008; NRC 2007). Diese Vorgehensweise führt derzeit zu einem Paradigmenwechsel in der Toxikologie. Sah der bisherige Ansatz die Exposition eines

Versuchstiers mit der Noxe und die Erfassung adverser Effekte vor, soll nun mithilfe von Methoden wie der Toxikogenomik, Bioinformatik, Systembiologie und der computergestützten Toxikologie an *in vitro* oder *in silico* Modellen der Wirkungspfad einer Noxe genauer untersucht und verstanden werden (Collins et al. 2008; Gibb 2008). In diesem Zusammenhang hat die U.S. EPA das groß angelegte Forschungsvorhaben „ToxCast“ initiiert, in dem anhand der Untersuchung von Bioaktivitätsprofilen und chemischen Eigenschaften einer Vielzahl von Chemikalien Methoden entwickelt werden sollten, die eine Priorisierung von Chemikalien für weiterführende Toxizitätstestungen zulassen (Dix et al. 2007; Judson et al. 2010). Abbildung 1-3 fasst diesen neuen Ansatz der Toxizitätstestung zusammen.

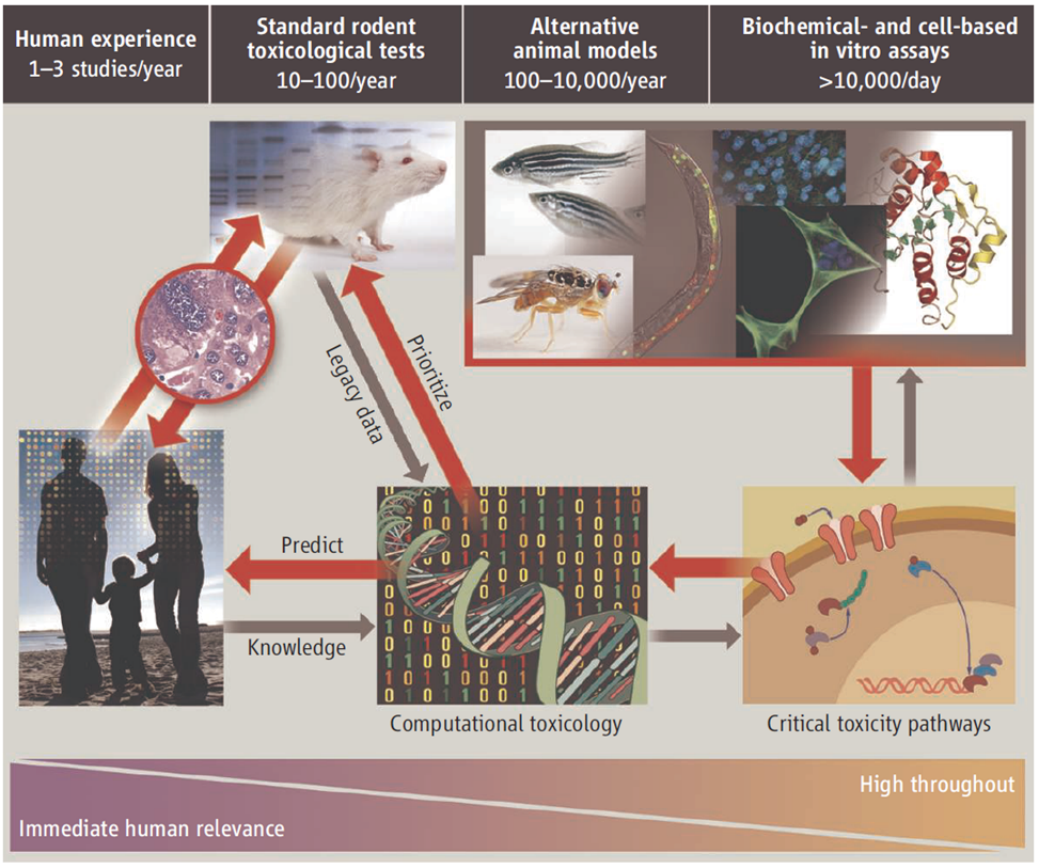

Abbildung 1-3: Paradigmenwechsel in der Toxikologie (Collins et al. 2008).
Hochdurchsatz-Assays und computergestützte Ansätze der Toxikologie sollen Daten liefern, die über den Aufschluss kritischer Toxizitäts-Wirkungspfade eine Priorisierung weiterer Tests im Tier ermöglichen und so eine Vorhersage des Risikos für den Menschen unterstützen.

Eine Herangehensweise, die einen Rahmen für die gesammelten Informationen und deren Rationalisierung bietet, ist das so genannte „Adverse Outcome Pathway“ (AOP) Konzept (Ankley et al. 2010). Nach diesem Konzept soll der adverse Effekt einer Substanz vom initialen molekularen Ereignis bis zum Einfluss auf den Organismus und am Ende auf die betroffene Population beschrieben werden (OECD 2013). Wie in Abbildung 1-4 dargestellt wer-

den ausgehend vom initialen Ereignis die darauf folgenden Ereignisse in den unterschiedlichen Ebenen der biologischen Organisation beschrieben.

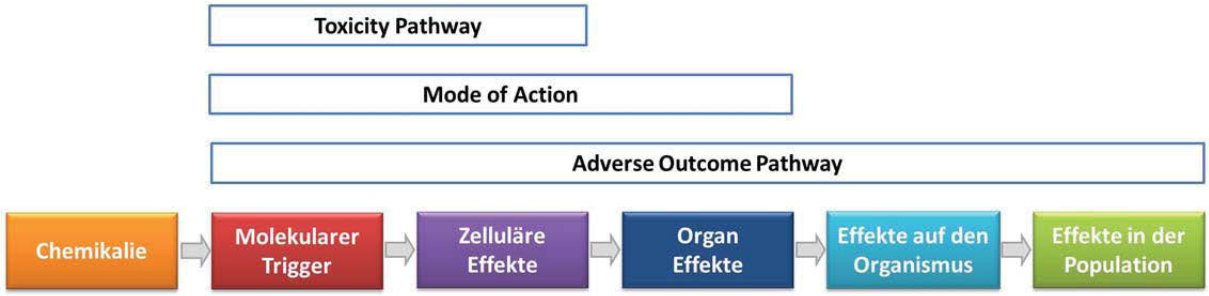

Abbildung 1-4: Schematische Darstellung des Adverse Outcome Pathway Konzeptes (modifiziert nach OECD 2013).
Dargestellt sind die unterschiedlichen Auswirkungen einer Interaktion des menschlichen Gewebes mit einer Noxe in den verschiedenen biologischen Organisationsebenen.

Das weit gefasste Ziel des AOP Konzeptes ist eine bessere und vor allem prädiktive Gefahrenidentifizierung sowie die verbesserte Klassifizierung der im Umlauf befindlichen Chemikalien. So sollen beispielsweise quantitative Struktur-Aktivitäts-Beziehung von Chemikalien hergestellt werden, die mit bestimmten initialen Ereignissen verbunden sind und damit eine frühzeitige Einschätzung der Toxizität erlauben. Über die Identifizierung bestimmter Schlüsselereignisse in einem AOP soll das Gefahrenpotential einer Chemikalie charakterisiert werden. Sind solche Schlüsselereignisse für einen bestimmten adversen Effekt aufgeklärt, können diese zur Identifizierung von Biomarkern dienen und für die Entwicklung neuer *in vitro* Toxizitätstests eingesetzt werden (Andersen et al. 2012; OECD 2013). Die *in vitro* Modelle, die bei der Untersuchung von Wirkmechanismen für eine Substanz innerhalb des AOP Konzeptes zum Einsatz kommen, sollten die (vorzugsweise menschliche) Physiologie so gut wie möglich abbilden. Im Hinblick auf die Untersuchung von Speziesunterschieden zwischen der Ratte, dem vorherrschenden Tiermodel in der Toxikologie, und dem Menschen sollten außerdem korrespondierende *in vitro* Modelle beider Spezies generiert werden können, um so einen direkten Vergleich von qualitativen und quantitativen Antworten auf eine Chemikalienexposition zu ermöglichen. Damit soll die Unsicherheit in der Einschätzung des Gefahrenpotentials durch die Erhebung spezies-spezifischer mechanistischer Daten für einzelne Chemikalien reduziert werden (Bal-Price et al. 2015a).

Die Entwicklung von AOPs für Entwicklungsneurotoxizität ist besonders herausfordernd, da die Prozesse während der Gehirnentwicklung sehr komplex sind, die Anfälligkeit des sich entwickelnden Nervensystems auf Toxine vom jeweiligen Entwicklungsstadium des Gehirns

abhängt und die Pathophysiologie vieler Neuroentwicklungsstörungen nur wenig verstanden ist. Aus diesen Gründen ist die Verknüpfung molekularer initialer Ereignisse und neurologischer Entwicklungsstörungen für nur wenige Chemikaliengruppen bekannt (Bal-Price et al. 2015b), was die Entwicklung alternativer Ansätze für die Toxizitätstestung erschwert.

1.4. Primäre neurale Progenitorzellen als *in vitro* Modell für Entwicklungsneurotoxizitätstestung

Aktuell stehen noch keine validierten *in vitro* Assays für die Untersuchung von Entwicklungsneurotoxizität zur Verfügung. In den letzten Jahren wurden jedoch große Anstrengungen unternommen, zellbasierte Teststrategien für die Charakterisierung des entwicklungsneurotoxischen Gefährdungspotentials von Chemikalien zu entwickeln. Bisher wurden vor allem Primärzellen aus dem Nager und alternative Modelorganismen wie der Zebrafisch hinsichtlich ihrer Eignung für Entwicklungsneurotoxizitätstestung untersucht (Coecke et al. 2007). Dessen größte Limitierung besteht jedoch - wie auch bei der Testung in der Ratte - in den Speziesunterschieden zum Menschen. Weiterhin wurde eine Reihe von Tumorzelllinien für die Testung auf Entwicklungsneurotoxizität eingesetzt (Harrill and Mundy 2011; Scholz et al. 2011; Stern et al. 2014), ein großer Nachteil der immortalisierten Zelllinien ist allerdings die Entartung der Zellen und die damit fehlende Übertragbarkeit auf normale Zellen. Dem gegenüber stehen Stamm- und Progenitorzellen, die primären Ursprungs sind, aus dem Menschen gewonnen werden können und damit zunehmend an Bedeutung für Entwicklungsneurotoxizitätstestungen gewinnen (Baumann et al. 2014; de Groot et al. 2013; Hayess et al. 2013).

Normale primäre neurale Progenitorzellen (NPCs) können *in vitro* basale Prozesse der Gehirnentwicklung abbilden (Baumann et al. 2014; Fritsche et al. 2011; Moors et al. 2009). Weiterhin können sie als dreidimensionale Zellaggregate kultiviert werden und erweisen sich damit als 3D Ko-Kultursystem als besonders geeignet, da so die physiologischen Bedingungen im Vergleich zu zweidimensional kultivierten Zellsystemen besser erhalten bleiben (Yamada and Cukierman 2007). Bei Progenitorzellen handelt es sich um Vorläuferzellen, die bereits auf ein bestimmtes Zielorgan determiniert sind, aber den auf die Regenerationseigenschaft bezogenen Stammzellcharakter behalten. NPCs können aus den Vollhirnhomogenaten menschlicher oder Nager-Feten bzw. postnataler Nagerjungen gewonnen werden. In Suspensionskultur und in Gegenwart von Wachstumsfaktoren (Epidermal Growth Factor, EGF, und Fibroblast Growth Factor, FGF) kultiviert, bilden sie spontan dreidimensionale Zel-

laggregate aus, die als Neurosphären bezeichnet werden (Buc-Caron 1995; Chalmers-Redman et al. 1997; Reynolds et al. 1992; Svendsen et al. 1995). Die Neurosphären können bis zu mehreren Monaten unter Erhalt ihrer proliferativen Eigenschaften in Suspensionskultur gehalten und aufgrund ihrer proliferativen Größenzunahme durch mechanische Zerkleinerung passagiert werden (Svendsen et al. 1997).

Neben ihrer proliferativen Eigenschaft besitzen NPCs in Gegenwart einer extrazellulären Matrix und bei Entzug von Wachstumsfaktoren die Fähigkeit der radialen Migration und Differenzierung. Sie bilden eine heterogene Zellpopulation, die Marker neuraler Progenitorzellen (Nestin$^+$) sowie charakteristische Proteine der drei im Gehirn vorherrschenden Zelltypen, nämlich Neuronen (β-(III)-Tubulin$^+$), Astrozyten (GFAP$^+$) und Oligodendrozyten (O4$^+$) exprimiert (Brannen and Sugaya 2000; Lobo et al. 2003; Piper et al. 2001; Reubinoff et al. 2001). Auch das Verhältnis zwischen Neuronen und Gliazellen in der Migrationsfläche hat sich als weitestgehend physiologisch erwiesen (Baumann et al. 2014). Weiterhin sind NPCs in der Lage, in die caspase-abhängige und -unabhängige Apoptose zu gehen (Moors et al. 2009), und können so zellzahlregulierende Prozesse während der Hirnentwicklung nachbilden. Folgende Abbildung 1-5 fasst das Prinzip der Neurosphärengenerierung zusammen.

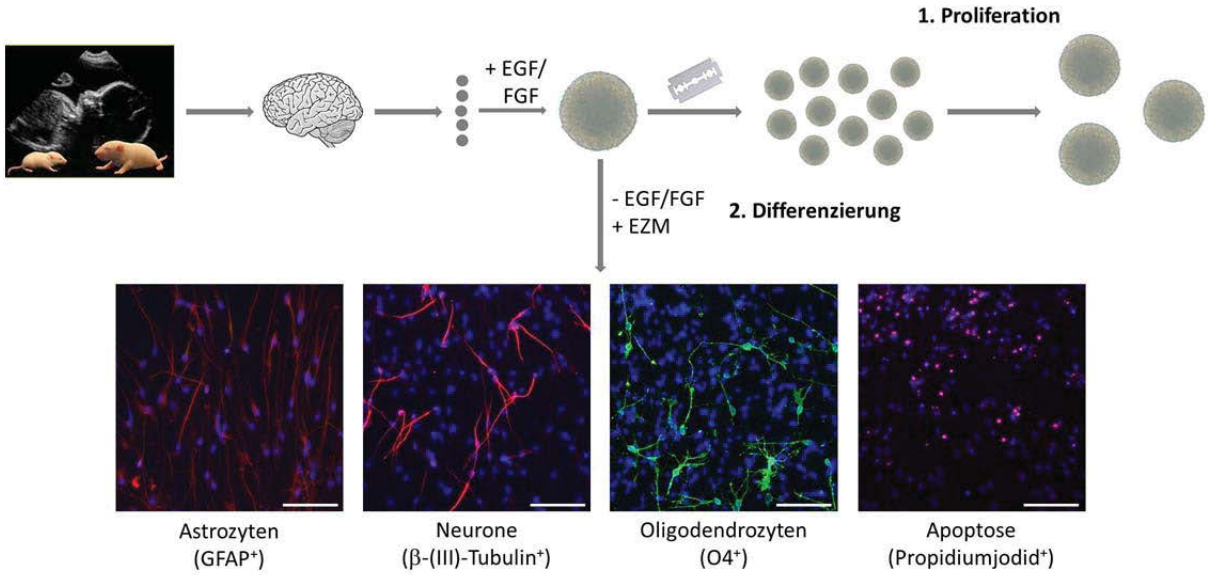

Abbildung 1-5: Das Prinzip der *in vitro*-Generierung von Neurosphären.
Aus Vollhirnhomogenaten werden neurale Progenitorzellen gewonnen, die in Gegenwart von Wachstumsfaktoren (EGF, FGF) kultiviert werden und Neurosphären ausbilden. 1. Neurosphären haben unter diesen Bedingungen proliferative Eigenschaften und können durch mechanische Zerkleinerung passagiert werden. 2. Bei Entzug der Wachstumsfaktoren und Anbieten einer Extrazellulärmatrix differenzieren sie zu den vorherrschenden neuralen Zelltypen des Gehirns und können in die Apoptose gehen. Abgebildet sind humane Neurosphären unter proliferierenden Bedingungen, immunzytochemische Färbungen differenzierter Astrozyten (rot), Neurone (rot) und Oligodendrozyten (grün) sowie eine Propidiumjodid-Färbung (rot) humaner Neurosphären (in blau Hoechst Färbung der Zellkerne). Längenmaßstab = 100 µm.

Somit stellen Neurosphären ein dreidimensionales Ko-Kultursystem dar, das aus den im Gehirn vorherrschenden Zelltypen in weitestgehend physiologischem Verhältnis besteht und die basalen Prozesse der Gehirnentwicklung, nämlich Proliferation, Migration, Differenzierung und Apoptose abbildet. Damit bietet es ein geeignetes *in vitro* System für die Entwicklungsneurotoxizitätstestung. Im sogenannten „Neurosphärenassay“ werden routinemäßig diese bedeutenden Prozesse der Gehirnentwicklung nach Exposition mit einer Testsubstanz untersucht. Dafür werden die Neurosphären zunächst mechanisch zerkleinert („gechoppt“), um eine homogene Sphärenpopulation einer definierten Größe zu generieren. Drei Tage später werden die Neurosphären unter proliferierenden Bedingungen, d.h. als freischwimmende Neurosphären, oder differenzierenden Bedingungen, d.h. unter Wachstumsfaktorentzug auf einer extrazellulären Matrix wachsend, gegenüber einer Chemikalie exponiert, um nach ein bis drei Tagen Effekte auf neuroentwicklungsrelevante Endpunkte sowie die allgemeine Viabilität der Zellen zu untersuchen. Änderungen im Proliferationsverhalten der Neurosphären werden mit Hilfe des BrdU-Assays, der die DNS-Synthese misst, detektiert, die Migration wird durch das Messen der Migrationsstrecke zwischen dem Sphärenkern und dem am weitesten migrierten Zellen quantifiziert, und die Differenzierung neuraler Progenitorzellen zu Neuronen innerhalb der Migrationsfläche wird durch immunzytochemische Anfärbung des neuronenspezifischen Zytoskelettproteins β-(III)-Tubulins untersucht. Gleichzeitig misst der Alamar Blue Assay über eine Quantifizierung der mitochondrialen Atmungskettenaktivität jeweils die Viabilität der Zellen, wodurch spezifische Effekte einer Chemikalie auf Proliferation, Migration oder Differenzierung und unspezifische Effekte auf die Zellviabilität unterschieden werden können. Die Testung einer Chemikalie im Neurosphärenassay kann so dazu beitragen, erste Hinweise auf das entwicklungsneurotoxische Potential einer Chemikalie zu erhalten und so Substanzen für weiterführende Untersuchungen zu priorisieren. Abbildung 1-6 fasst den Aufbau des Neurosphärenassays für die Entwicklungsneurotoxizitätstestung zusammen.

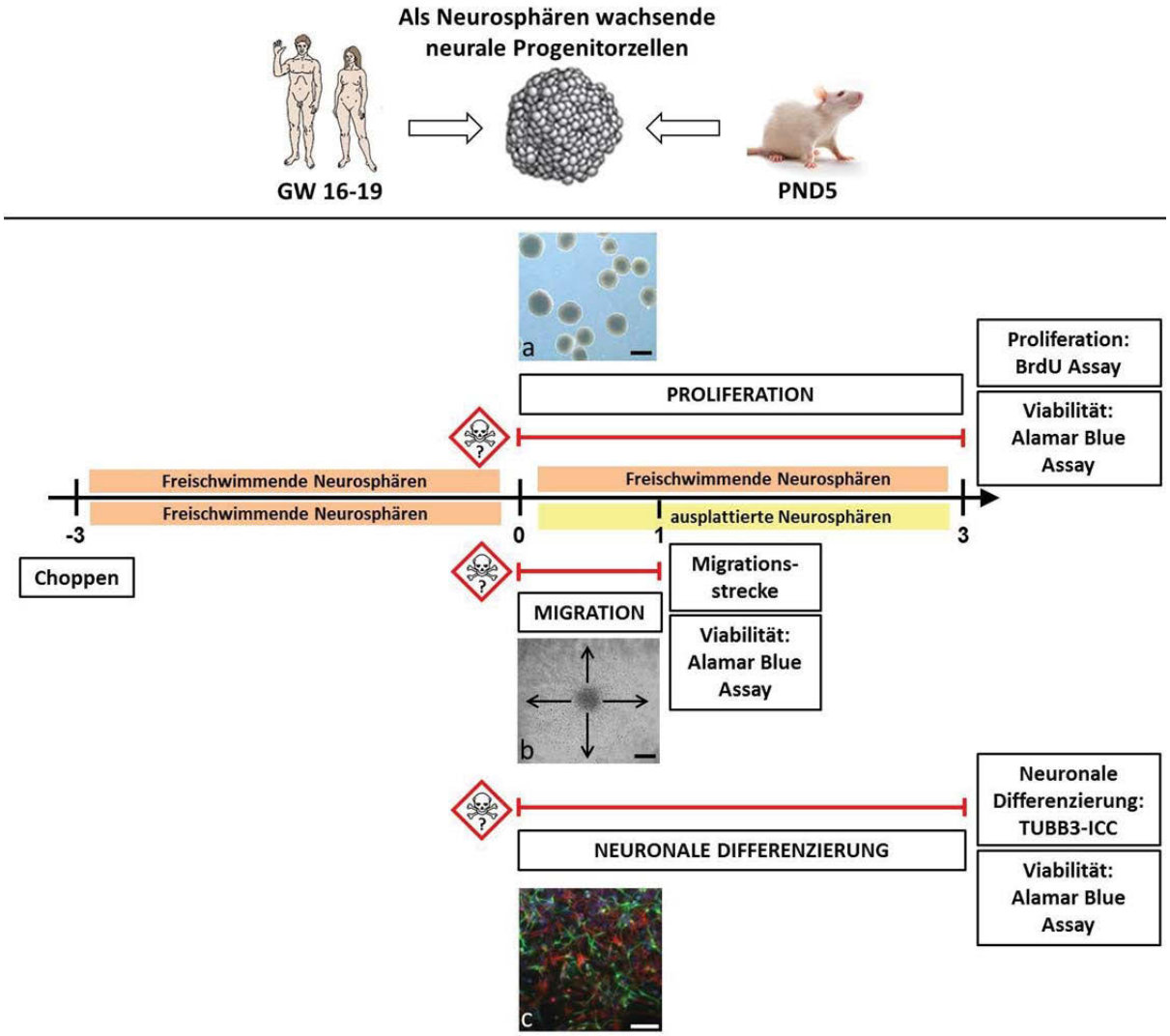

Abbildung 1-6: Chemikalientestung mit Hilfe des Neurosphärenassays.
Humane und Rattenneurosphären werden entweder als freischwimmende Sphären oder als auf einer extrazellulären Matrix ausplattierte Sphären mit einer Testchemikalie exponiert (gekennzeichnet in rot). Nach einem Tag wird das Migrationsverhalten analysiert und nach drei Tagen werden Proliferation und neuronal Differenzierung untersucht. Die Viabilität der Neurosphären wird parallel zu jedem der drei Endpunkte bestimmt. Zeitskala in Tagen. Längenmaßstäbe a und b 300 µm, c 100 µm. c. Rot: GFAP positive Zellen, grün: β-(III)-Tubulin positive Zellen, blau: Zellkerne.

Im Hinblick auf die Problematik der Speziesunterschiede für die Chemikalienrisikobewertung bietet das Neurosphärensystem den großen Vorteil, dass Neurosphären aus verschiedenen Spezies gewonnen werden können (humane Neurosphären, Nager-Neurosphären). Dabei ist der Vergleich der Gehirnentwicklung zwischen Nager und Mensch für eine zuverlässige Interspeziesextrapolation von essenzieller Bedeutung. Mit Hilfe eines mathematischen Algorithmus, welcher Gemeinsamkeiten und Unterschiede im zeitlichen Ablauf bestimmter Vorgänge während der neuralen Entwicklung vergleicht, lassen sich Korrelationen zwischen den Zeitpunkten, zu denen ähnliche Entwicklungsstände während der Gehirnentwicklung erreicht

sind, treffen. So korrelieren zum Beispiel die Entwicklungsstände des menschlichen Gehirns der 16. Gestationswoche mit dem Rattenhirn am Tag fünf nach der Geburt (Clancy et al. 2007).

Im konventionellen Ansatz der Risikobewertung von Chemikalien auf die menschliche Gesundheit ist der Einsatz von Unsicherheitsfaktoren für die Extrapolation von Effekten im Tier auf den Menschen vorgesehen. Für diesen Unsicherheitsfaktor wird standardmäßig der Wert „zehn“ verwendet, der sich zu gleichen Teilen aus einem toxikodynamischen und toxikokinetischen Teil zusammensetzt (Burgess-Herbert and Euling 2013). Vergleichende Untersuchungen in humanen und Nagerneurosphären erlauben es, Speziesunterschiede direkt zu untersuchen. Ein Parallelogrammansatz ermöglicht, basierend auf einem *in vitro* Vergleich von humanen und Nagerdaten sowie unter Einbeziehung von *in vivo* Daten des Nagers, eine Extrapolation auf Effekte im Menschen (Baumann et al. 2015). Weiterhin könnten molekulare Untersuchungen von Speziesunterschieden und deren Einordnung in das AOP-Konzept helfen, den toxikodynamischen Anteil des Interspezies-Unsicherheitsfaktors anzupassen, und den Einsatz von Extrapolationsfaktoren, die auf Chemikalien und Toxizitätsmechanismen basieren, im Prozess der Risikobewertung einzusetzen (Abbildung 1-7).

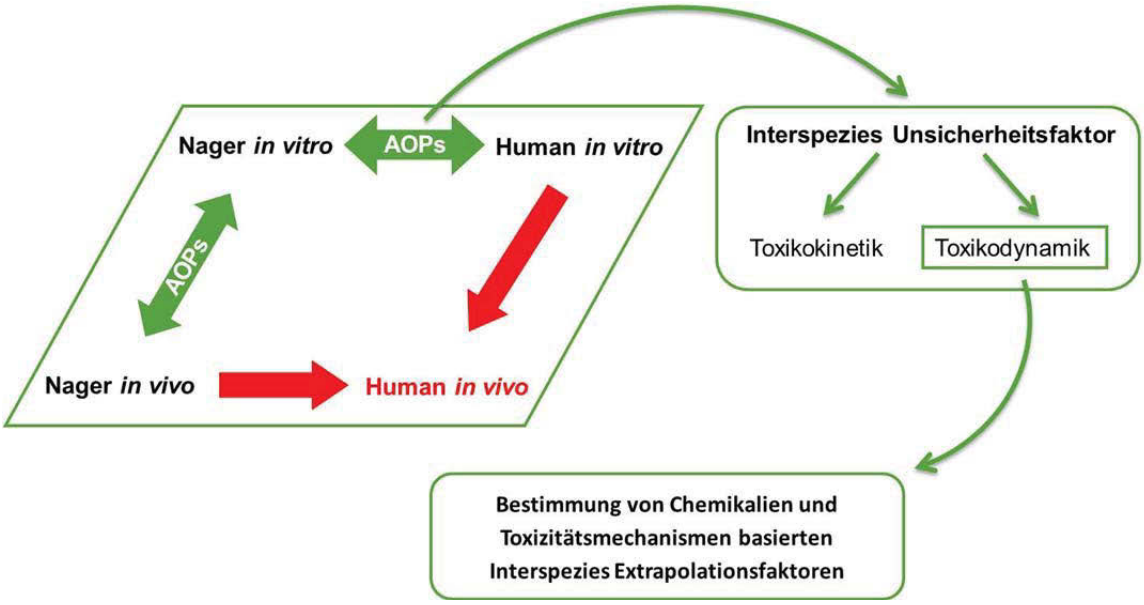

Abbildung 1-7: Chemikalien- und Toxizitätsmechanismen basierter Ansatz der Chemikalienrisikobewertung.
In einem Paralellogrammansatz werden *in vivo* Daten von Nagern mit *in vitro* Daten von Nagern verglichen, was *in vitro-in vivo* Ähnlichkeiten bzw. Unterschiede verdeutlicht. Nager *in vitro* Daten werden mit humanen *in vitro* Daten in äquivalenten Zellsystemen verglichen, wodurch Informationen zu Interspezies-Unterschieden gewonnen werden können. All diese Daten erlauben eine Extrapolation auf mögliche humane Effekte *in vivo*. Grün: Experimentelle Daten, Rot: Extrapolation. Die molekulare Untersuchung von Speziesunterschieden innerhalb des AOP-Konzeptes ermöglicht dabei eine Anpassung des toxikodynamischen Teils des Interspezies-Unsicherheitsfaktors.

Da in den klassischen Tierversuchsstudien gängige Rattenstämme wie die Wistar Ratte oder Sprague Dawley Ratte als bevorzugtes Versuchstier verwendet werden (OECD 2007; USEPA 1998), wurden in der vorliegenden Dissertation vorwiegend Neurosphären der Wistar Ratte verwendet. Neben der Untersuchung von Speziesunterschieden ermöglicht eine vergleichende Chemikalientestung in humanen und Rattenneurosphären eine Validierung des Neurosphärenassays für Entwicklungsneurotoxizitätsscreening im Hinblick auf *in vitro* - *in vivo* Vergleichbarkeiten (Baumann et al. 2015). Zum jetzigen Zeitpunkt sind nur 12 Chemikalien bekannt, die nachgewiesenermaßen entwicklungsneurotoxisch für den Menschen sind (Grandjean and Landrigan 2014), was eine Untersuchung der Vorhersagekraft des Neurosphärenassays für das entwicklungsneurotoxische Potential von Chemikalien erschwert. Für die Ratte hingegen existieren deutlich mehr Daten aus zahlreichen *in vivo* entwicklungsneurotoxischen Untersuchungen der letzten Jahrzehnte (Crofton et al. 2011). Für eine Festlegung der biologischen Applikationsdomäne des Neurosphärenassays ist also – sofern keine humanen Daten für eine Chemikalie verfügbar sind – eine vergleichende Untersuchung in humanen und Rattenneurosphären hilfreich, um die Vorhersagekraft der humanen *in vitro* Daten für die *in vivo* Situation einschätzen zu können. Für eine solche Validierung ist eine schnelle und effiziente Testung eines großen Chemikalientestsets unabdingbar (Crofton et al. 2011). Im Rahmen dieser Dissertation wurden erste Schritte für eine solche Validierung unternommen, in dem ein „Training Set“ von neun Chemikalien vergleichend in humanen und Rattenneurosphären getestet wurde.

1.5. Zielsetzung dieser Dissertation

Interessengruppen (Regulatoren, akademische Wissenschaftler, Industrievertreter) auf beiden Seiten des Atlantiks sind zu der Übereinkunft gekommen, dass sicherheitstoxikologische Testungen in der Chemikalienzulassung im Bereich von Entwicklungsneurotoxizität essentiell sind, wobei der Goldstandard für Toxizitätstestung nach wie vor der Tierversuch im Nager ist. Im Zusammenhang mit dem Paradigmenwechsel in der Toxikologie – weg von klassischen Tierversuchsstudien hin zu der genaueren Untersuchung des Toxizitätsmechanismus einer Noxe mit Hilfe von *in vitro* und *in silico* Methoden unter Berücksichtigung von Speziesunterschieden zwischen Versuchstier und Mensch – war es das übergeordnete Ziel dieser Dissertation, einen *in vitro* Screeningassay für Entwicklungsneurotoxizitätstestung basierend auf primären neuralen Progenitorzellen, der eine direkte Untersuchung von Speziesunterschieden zwischen Nager und Mensch zulässt, und dessen biologische Applikationsdomäne zu charakterisieren und molekulare Speziesunterschiede sowie deren Bedeutung für den

Prozess der Risikobewertung von Chemikalien für die Gesundheit des Menschen genauer zu untersuchen.

Zum Erreichen dieses Zieles wurden die folgenden Aufgabenstellungen bearbeitet:

1. Vertiefende Charakterisierung des Neurosphärenassays als dreidimensionaler *in vitro* Screening Assay für die Entwicklungsneurotoxizitätstestung von Chemikalien und Pharmazeutika

2. Untersuchung der Vorhersagekraft des Neurosphärenassays mittels Testung eines gut charakterisierten Training Sets an Chemikalien, die nachgewiesenermaßen entwicklungsneurotoxisches Potential haben (Positivsubstanzen: Methylquecksilberchlorid, Natriumarsenit, Chlorpyrifos, Parathion, Methylazoxymethanolazetat und Natriumvalproat) sowie solchen, die die Gehirnentwicklung *in vivo* nicht beeinträchtigen (Negativsubstanzen: Natriumglutamat, Paracetamol und Penicillin G) in humanen und Rattenneurosphären

3. Untersuchung der molekularen Mechanismen, die der spezies-spezifischen Empfindlichkeit auf entwicklungsneurotoxische Substanzen zu Grunde liegen, anhand des gut charakterisierten und für den Menschen entwicklungsneurotoxischen Antiepileptikums Natriumvalproat (Ornoy 2009)

2. Manuskripte

Im Folgenden sind die Publikationen, die aus dieser Dissertation hervorgingen, angefügt. Bei der ersten Publikation (2.1) handelt es sich um ein Buchkapitel mit dem Titel „Comparative Human and Rat 'Neurosphere Assay' for Developmental Neurotoxicity Testing". In dieser Publikation wird die Untersuchung des entwicklungsneurotoxischen Potentials von Chemikalien im Neurosphärenassay von Ratte und Mensch genau beschrieben und ausführliche Protokolle für die Neurosphärenkultivierung und Chemikalientestung gegeben. Nach dieser generellen Einführung in den Neurosphärenassay wird in der darauffolgenden Publikation 2.2 (Originalarbeit: „Automated neurosphere sorting and plating by the COPAS large particle sorter is a suitable method for high-throughput 3D *in vitro* applications") eine Methode beschrieben, die den Sortier- und Plattiervorgang der Neurosphären für die Assays automatisiert und damit eine Durchsatzerhöhung in der Chemikalientestung ermöglicht. In Publikation 2.3 (Buchkapitel: „Application of the Neurosphere Assay for DNT Hazard Assessment: Challenges and Limitations") werden schließlich humane und Nagerneurosphären (Maus und Ratte) unter dem Aspekt von Speziesunterschieden, die für die regulatorische Chemikalienrisikobewertung von Bedeutung sind, verglichen und die physiologische Relevanz des Zellmodells für die *in vivo* Gehirnentwicklung untersucht. Die Ergebnisse dieser Publikation gaben die Grundlage dafür, in Publikation 2.4 (Originalarbeit: „Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events") den Neurosphärenassay von Ratte und Mensch auf seine Vorhersagekraft für das entwicklungsneurotoxische Potential eines Chemikalien-Trainingsets zu überprüfen und in einem Parallelogrammansatz *in vitro* und *in vivo* Ergebnisse von Mensch und Ratte miteinander zu vergleichen. Dabei ergaben sich signifikante Speziesunterschiede zwischen Ratten- und humanen Neurosphären hinsichtlich ihrer Sensitivität auf die Testchemikalien, weshalb in Publikation 2.5 (Originalarbeit: „21st Century Risk Assessment: case study for species-specific hazard characterization of valproic acid") die molekularen Mechanismen, die dem Speziesunterschied in der Sensitivität gegenüber dem Antiepileptikum Natriumvalproat zu Grunde liegen, untersucht und in den Kontext von Konzepten der modernen Risikobewertung des 21. Jahrhunderts gestellt wurden.

2.1. Comparative Human and Rat "Neurosphere Assay" for Developmental Neurotoxicity Testing

J. Baumann, M. Barenys, K. Gassmann, E. Fritsche

Current Protocols in Toxicology [zur Publikation angenommen am 04. Dezember 2013]

Das sich entwickelnde Nervensystem reagiert sehr empfindlich auf die adversen Effekte von Chemikalien. Im Zuge der neuen Chemikalienverordnung REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) wächst der Bedarf für die Testung und Regulierung von Chemikalien des allgemeinen Gebrauchs und, aufgrund des bisherigen Datenmangels, für die Identifizierung von Entwicklungsneurotoxinen. In diesem Zusammenhang werden alternative Teststrategien benötigt, die ein schnelles und kostengünstiges Screenen von Chemikalien ermöglichen und die Anzahl an Tierversuchen auf ein Minimum reduzieren. In diesem Kapitel stellen wir ein dreidimensionales *in vitro* Model für Entwicklungsneurotoxizitätsscreening, das auf primären neuralen Progenitorzellen von Mensch und Ratte basiert, vor. Dieses Zellmodell kann Störungen in basalen Prozessen der Gehirnentwicklung, wie Proliferation, Migration, Differenzierung und Apoptose detektieren und erlaubt eine Abgrenzung dieser spezifischen Chemikalieneffekte von genereller Zytotoxizität. Wir beschreiben Protokolle (i) für die Generierung und Kultivierung humaner und Rattenneurosphären, (ii) für die Untersuchung der neuroentwicklungsrelevanten sowie Zytotoxizitätsendpunkte und (iii) für eine Auswertung der erhobenen Daten, um festzustellen, ob eine getestet Substanz entwicklungsneurotoxisches Potential hat. Der Vergleich von humanen und Rattendaten ermöglicht dabei eine direkte Untersuchung von Speziesunterschieden in der Toxikodynamik von Chemikalien und trägt damit zu einer Verbesserung der humanen Risikobewertung von Entwicklungsneurotoxinen bei.

Comparative Human and Rat "Neurosphere Assay" for Developmental Neurotoxicity Testing

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[2]Both authors contributed equally to this unit

ABSTRACT

The developing nervous system is highly vulnerable to the adverse effects of chemical agents. Currently, there is an increasing need for testing and regulating chemical compounds in general use and, due to the lack of available data, to identify those which are developmental neurotoxicants. In this context, alternative testing strategies are needed in order to allow fast and cost-efficient screening and to reduce the number of animal experiments usually required. In this unit we present an in vitro three-dimensional model for developmental neurotoxicity screening based on human and rat neural progenitor cells. This model enables the detection of disturbances in basic processes of brain development, such as proliferation, migration, differentiation and apoptosis, and allows the distinction of these specific disturbances from general cytotoxicity. Furthermore, the comparison of human and rat data provides useful insights into species differences for toxicodynamics of compounds contributing to human risk assessment of developmental neurotoxicants. *Curr. Protoc. Toxicol*. 59:12.21.1-12.21.24. © 2014 by John Wiley & Sons, Inc.

Keywords: developmental neurotoxicity • neural progenitor cell • in vitro • brain development • species differences

INTRODUCTION

Developmental neurotoxicity (DNT) is a serious threat to human health. It describes any adverse effect of exogenous noxae on the developing nervous system, leading to functional and morphological changes of the brain (Goldman and Koduru, 2000). Currently, animal experiments in rats are used for DNT testing, which is very expensive in terms of both cost and time and uses large numbers of animals, and bears the additional problem of species differences for extrapolation (Lein et al., 2005, 2007; Coecke et al., 2007). In this unit we present an in vitro method to assess DNT, which is based on a three-dimensional (3-D) cell culture model. Therefore, we use human and rat neural progenitor cells (NPC) growing as 3-D cell aggregates called neurospheres.

With the "Neurosphere Assay," we assess effects of chemicals on basic processes of brain development (e.g., proliferation, migration, differentiation, and apoptosis) to determine their developmental neurotoxic potential (Fritsche et al., 2005; Moors et al., 2007, 2009; Breier et al., 2010; Gassmann et al., 2010; Schreiber et al., 2010). Thereby, we expect that a compound is hazardous for brain development if only one of these endpoints is altered. Using both rat and human neurospheres gives us the opportunity to investigate toxicodynamic species differences in vitro, which can be further applied in toxicological risk assessment for DNT. Apart from the two species presented in this unit, mouse neurospheres are also a well-recognized model for DNT testing. For more details on mouse neurosphere cultivation, readers are referred to Fritsche et al. (2011).

An overview of all protocols presented in this unit is given in Figure 12.21.1. Basic Protocol 1 describes the steps for thawing and expanding commercially available human NPC and for isolating NPC from postnatal day (PND) 5 rat brains. Moreover, it explains

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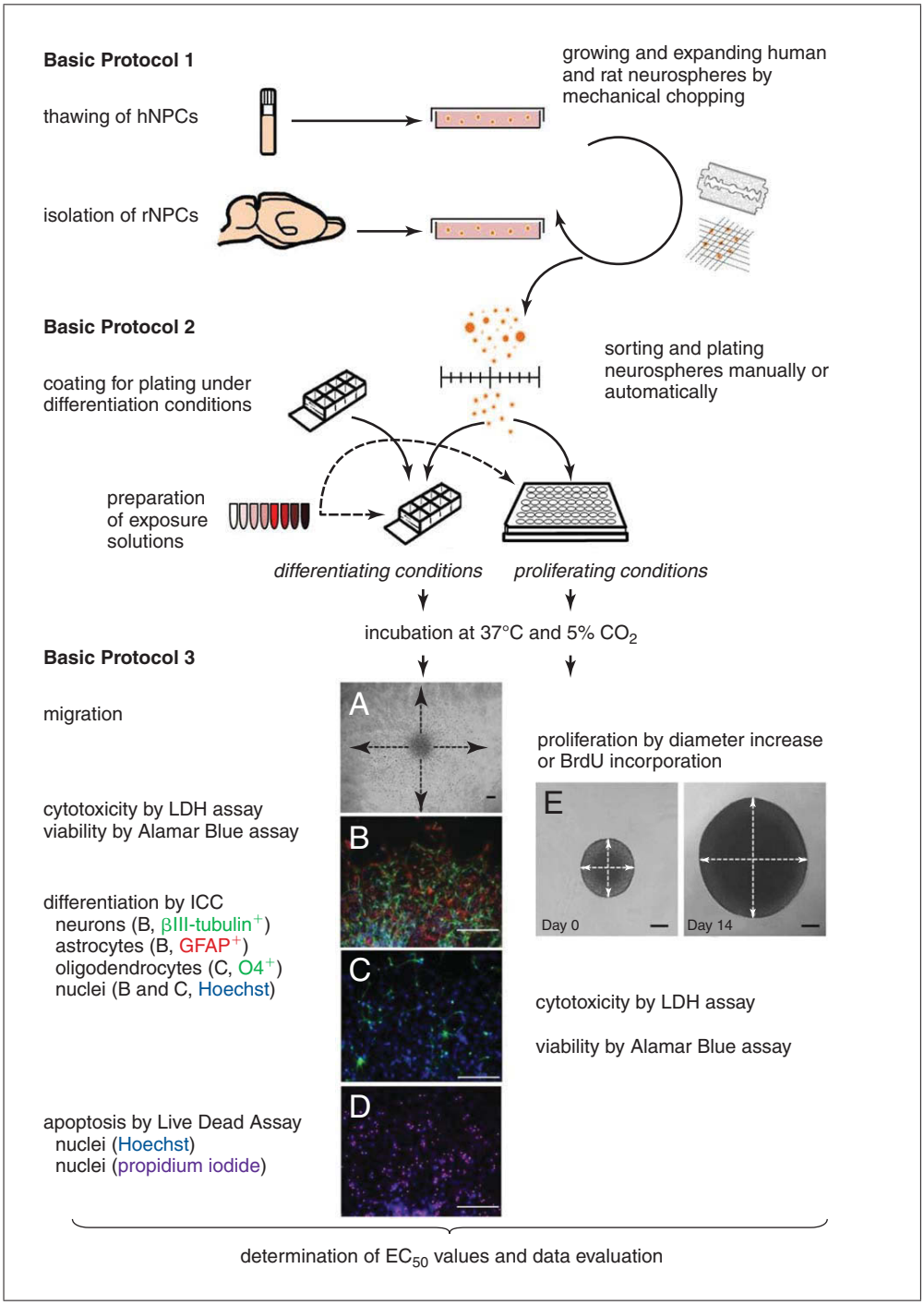


Figure 12.21.1 Schematic representation of the "Neurosphere Assay" for DNT testing. Basic Protocol 1 details the thawing and isolation of human and rat neural progenitor cells (NPCs) and the conditions to culture and expand them as neurospheres. Basic Protocol 2 explains the steps needed to start the "Neurosphere Assay" under differentiating and proliferating conditions: coating of 8-chamber slides, preparation of the exposure solutions, sorting of 0.3 mm neurospheres, and plating. Basic Protocol 3 describes the procedures to analyze the developmental neurotoxic endpoints: (**A**) migration measurement as indicated by dashed arrows, (**B**) differentiation evaluation by immunocytochemistry (ICC) with βIII-tubulin in green, GFAP in red, and Hoechst staining in blue, (**C**) differentiation evaluation by ICC with O4 in green and Hoechst staining in blue, (**D**) apoptosis assessment by Live Dead Assay with Hoechst staining in blue and propidium iodide in purple, (**E**) proliferation analysis by diameter increase as indicated by dashed arrows or by BrdU incorporation. Viability and cytotoxicity of differentiating and proliferating neurospheres is assessed to distinguish between cytotoxicity and specific developmental neurotoxicity. Scale bars: 100 μm.

how to cultivate and propagate human and rat neurospheres for several weeks to months. In Basic Protocol 2 we describe how to plate and expose human and rat neurospheres with compounds to assess effects on neurodevelopment and viability. We present two ways of plating neurospheres: (1) manual plating and (2) automated plating with a COPAS Large Particle Sorter. Basic Protocol 3 outlines the steps needed to perform the assays to analyze effects on the endpoints proliferation, migration, differentiation to neural effector cells, and apoptosis, as well as effects on viability and cytotoxicity (1) to determine if the test compound shows effects on neurodevelopment and (2) to check if those can be distinguished from general (or cell type-specific) cytotoxicity of the compound. Finally, we show one of the possible ways to evaluate the data obtained in Basic Protocol 3 to decide whether the compound of interest is developmentally neurotoxic by calculating EC_{50} values for each endpoint in both species.

NOTE: All protocols involving patient tissues require IRB approval and donor consent. Protocols employing live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to government regulations regarding the care and use of laboratory animals.

CULTIVATION OF HUMAN AND RAT NPCs AS NEUROSPHERES

BASIC PROTOCOL 1

Here we present how to generate and cultivate human and rat NPCs in suspension culture as 3-D neurospheres. While human NPCs are commercially available as whole brain cell suspensions from different donors of gestational weeks 16 to 20, we isolate rat NPCs from postnatal day (PND) 5 pups by dissecting, digesting, and homogenizing whole brains to obtain a cell suspension. Within one week, neurospheres of ~0.2 to 0.3 mm in diameter are formed.

Both rat and human neurospheres grow in size by cultivation in the presence of growth factors. By mechanical passaging with a razor blade (chopping), the neurospheres are cut into small pieces, regenerate, form regular smaller neurospheres of a uniform size within 1 or 2 days, and continue to grow in size. In this way, neurospheres can be expanded and cultured over several weeks (rat neurospheres) or months (human neurospheres) without losing their proliferative capacity.

Materials

Phosphate-buffered saline with Ca^{2+} and Mg^{2+} (PBS; Gibco, Life Technologies GmbH), sterile
Minimal essential medium (MEM; Biochrom AG)
PND 5 rat pups (in this protocol, the delivery day of the pups is considered as PND 0)
Tissue digestion solution (see recipe)
Dulbecco's modified Eagle medium (DMEM; Gibco GlutaMAX High Glucose, Life Technologies GmbH)
Ovomucoid solution (see recipe)
Proliferation medium (see recipe)
Cryovial of normal human neural progenitor (NHNP) cells (Lonza)
70% (v/v) ethanol
100% acetone

6- and 10-cm petri dishes
15- and 50-ml tubes
Scissors
Forceps
37°C incubator
1000-µl pipet tips
Centrifuge

McIlwain tissue chopper
Double-edged razor blade

NOTE: All procedures should be performed in a laminar-flow hood.

Isolation of rat NPCs

1a. Fill one petri dish with 1× PBS and one with MEM.

2a. Fill a 15-ml tube with 1 ml MEM.

3a. Decapitate the rat pup with a scissor.

4a. Place the head in a PBS-filled petri dish and remove the skin and cartilage with forceps to uncover the brain. Transfer the brain from the skullbase to the petri dish filled with MEM.

5a. Remove the meninges from the brain and collect the tissue in the 15-ml tube.

6a. Add 1 ml tissue digestion solution to the brain, cut it in small pieces with a scissor, and incubate for 30 min at 37°C

7a. Fill a 15-ml tube with 9 ml DMEM.

8a. Triturate the tissue gently with a 1000-µl pipet tip without producing air bubbles.

9a. Add 1 ml Ovomucoid solution to stop the digestion.

10a. Transfer the single-cell solution into the 15-ml tube filled with DMEM (from step 7a).

11a. Centrifuge the tube for 5 min at 170 × *g*, room temperature.

12a. Carefully discard the supernatant and resuspend the pellet in 1 ml proliferation medium.

13a. If more than one brain is prepared, pool the suspensions of all brains after resuspending the pellets in one tube and equally distribute the suspension into 10-cm petri dishes filled with 20 ml proliferation medium (one petri dish per brain).

14a. After 2 to 3 days, small neurospheres are formed. At this point, feed the cultures by replacing half of the medium with fresh proliferation medium.

During the first feeding of the cultures, replace the petri dish because there are usually differentiated brain cells or foreign cells (e.g., fibroblasts) adhered, which are not desirable in the culture. In addition, remove remaining undigested culture pieces (e.g., from blood vessels which were left prior digestion) from the culture.

Thawing of human NPCs

1b. Precondition 100 ml proliferation medium at 37°C and 5% CO_2.

2b. For thawing one cryovial of NHNP cells, distribute 50 ml preconditioned proliferation medium into five 10-cm petri dishes.

3b. Add the other 50 ml of preconditioned proliferation medium into a 50-ml tube.

4b. Place the cryovial in a 37°C water bath very briefly, just until the cells have started to thaw (~2 min).

5b. Add the content of the cryovial into the 50 ml tube (from step 3b) and resuspend the cells by gently pipetting up and down.

6b. Transfer the cell suspension into the 10-cm petri dishes (10 ml each).

7b. Place the petri dishes with the cell suspension into the cell culture incubator at 37°C and 5% CO_2.

8b. Feed the cell suspension every 2 days by replacing half of the medium with fresh proliferation medium.

After 4 weeks of expansion the human NPCs have built neurospheres of about 0.5 mm in diameter and can be used for experiments.

9b. Proceed with step 15.

Grow the human and rat neurospheres

15. Feed human and rat neurospheres every 2 to 3 days by replacing half of the medium with fresh proliferation medium.

Expand the human and rat neurospheres by mechanical chopping

To increase growth and survival, human and rat neurospheres should be chopped when they reach a diameter of 0.5 mm (for rat neurospheres) or 0.7 mm (for human neurospheres).

16. Place the McIlwain tissue chopper into a laminar flow culture hood and clean with 70% ethanol.
17. Soak a double-edged razor blade in 100% acetone and sterilize the sliding table and the chopping arm with 70% ethanol.
18. Carefully secure the blade onto the chopping arm. Make sure the blade is parallel to the chopping surface.
19. Check the chopper settings.

The blade force should be set at 12:00 (straight up), and, for optimal growth, the chop distance should be set between 0.15 and 0.25 mm.

20. Prepare 10-cm petri dishes for the newly chopped neurospheres by filling them with 20 ml proliferation medium each (usually two to three new petri dishes for chopping the neurospheres from one old petri dish).
21. Transfer the neurospheres with as little medium as possible from the old 10-cm petri dish into the middle of an inverted lid of a 6-cm petri dish.
22. Carefully remove the remaining medium with a pipet in order to prevent the neurospheres from moving during the chop.
23. Place the dish lid on the chopper and move the sliding table to the starting position.
24. Turn on the power, and press “reset.”
25. When all neurospheres on the lid have been chopped, stop and raise the chopping arm, and reposition the table on the starting position.
26. Rotate the dish lid 90° and repeat steps 24 and 25.
27. When the neurospheres have been chopped in the second direction, remove the dish lid from the chopper and add about 1 ml proliferation medium to the cells.
28. Resuspend the chopped neurospheres by gently pipetting them up and down and then equally distribute the cell suspension into the new petri dishes.
29. Put the cells back into the cell culture incubator.

We keep the dishes in the incubator until we need them the next time, either for feeding or for plating an experiment.

30. After chopping is complete, clean the chopper with 70% ethanol and eventually discard the razor blade (usually one blade can be used for three times each side).

Make sure the neurospheres are well distributed in the petri dish to avoid aggregation.

BASIC PROTOCOL 2

PLATING AND CHEMICAL EXPOSURE OF HUMAN AND RAT NEUROSPHERES

In this protocol we explain how to plate and expose human and rat neurospheres to chemicals in order to determine if a certain compound (1) disturbs their ability to proliferate, migrate, or differentiate into neural effector cells and/or (2) exerts cytotoxicity. Therefore, we distinguish exposure during two different differentiation states, proliferating and differentiating. For analyzing proliferation, neurospheres are kept as 3-D suspending aggregates in a 96-well round-bottom plate (proliferating conditions). For the differentiation state, during which we measure migration and differentiation, neurospheres are plated on a poly-D-lysine/laminin-coated surface in an 8-chamber slide. On this matrix, cells migrate out of the neurosphere forming a radial migration area.

The day of plating and exposing the neurospheres is considered as day 0 of the protocol. Prior to plating the neurospheres, serial dilutions of the chemical of interest, as well as endpoint-specific control solutions to monitor assay performance, need to be prepared, and for experiments under differentiating conditions 8-chamber slides need to be coated. For sorting and plating human and rat neurospheres, we propose two different methods. Neurospheres of a uniform size (1) can be sorted and plated manually with a 100-µl tip, either one sphere per 96-well or five spheres per chamber (Fig. 12.21.2) or (2) can be sorted with a COPAS Large Particle Sorter in an automated manner (Gassmann et al., 2012). Automated plating of one sphere per well in a 96-well plate is possible while positioning of five spheres per chamber, as shown in Figure 12.21.2, includes one manual positioning step after automated plating. Sorting and plating human and rat neurospheres in an automated way opens up the opportunity of medium-throughput compound testing, whereas manual sorting and plating is sufficient if only a few compounds need to be tested.

Materials

Poly-D-lysine (see recipe)
Sterile water
1 mg/ml laminin (Sigma-Aldrich, cat. no. L2020)
Phosphate-buffered saline with Ca^{2+} and Mg^{2+} (PBS; Gibco, Life Technologies GmbH), sterile
Proliferation medium (see recipe)
Differentiation medium (see recipe)
Test compound stock solution with respective solvent
Solutions for the endpoint-specific controls (Table 12.21.1)
Chopped human and rat neurosphere (from Basic Protocol 1)
Ethanol
Cleaning solution (for COPAS Large Particle Sorter; Union Biometrica)

8-chamber slides
37°C incubator
Round-bottom 96-well plates
6- and 10-cm petri dishes
100-µl tips
Binocular microscope
Micrometer
COPAS Large Particle Sorter (Union Biometrica)

NOTE: All procedures should be performed in a laminar-flow hood.

Coat 8-chamber slides for plating neurospheres under differentiating conditions

1. Fill every chamber of an 8-chamber slide with 250 µl poly-D-lysine and incubate for at least 1 hr at 37°C.

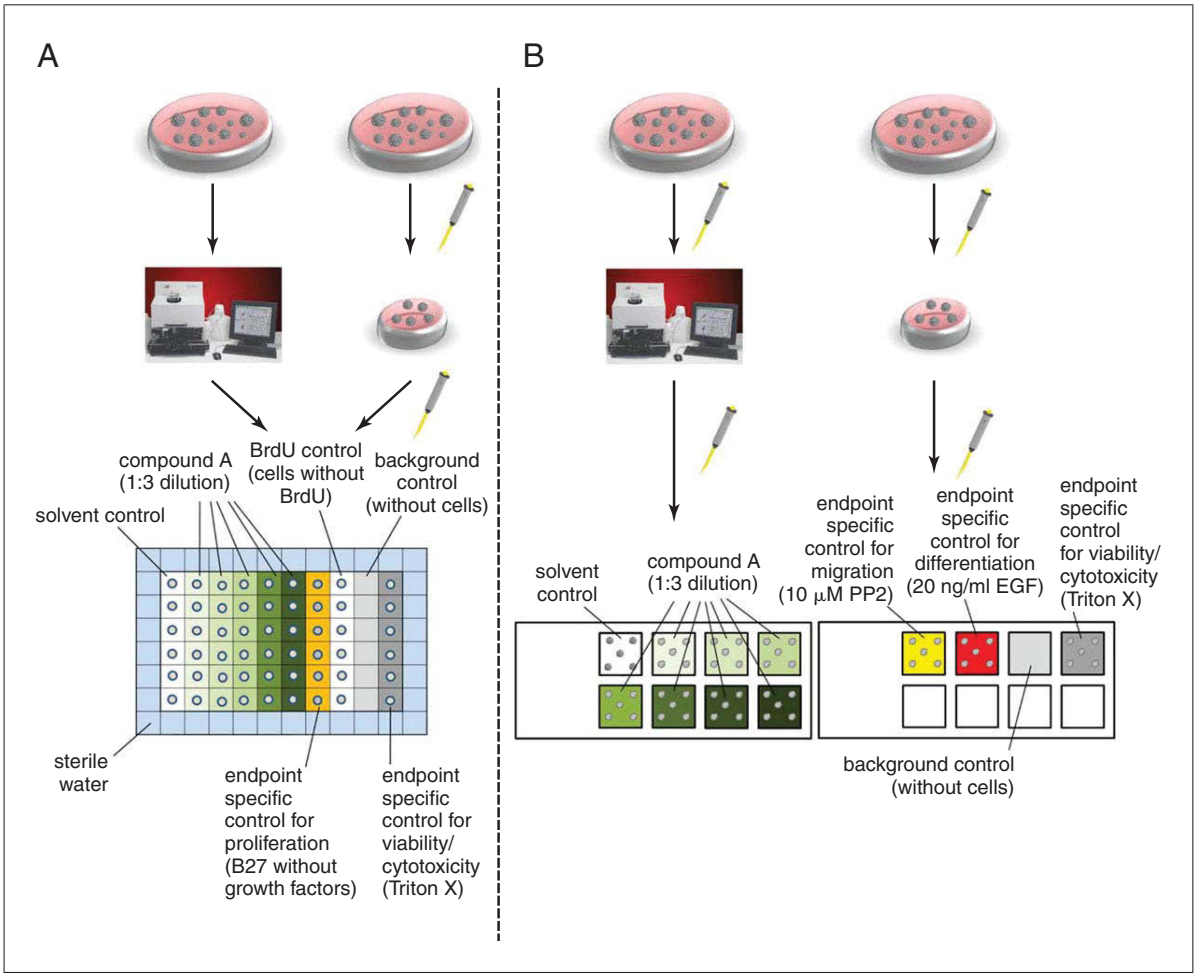


Figure 12.21.2 Plating of neurospheres. For experiments under proliferating conditions, neurospheres can be either sorted and plated automatically with a COPAS Large Particle Sorter (A) or manually with a 100-μl pipet (B). For experiments under differentiating conditions, neurospheres can be sorted with a COPAS Large Particle Sorter (A) or manually (B) and subsequently positioned manually in the chambers with a 100-μl pipet.

2. Wash every chamber with 500 μl sterile water.
3. Fill every chamber with 250 μl laminin (1:100 dilution of laminin in sterile water) and incubate for at least 1 hr at 37°C.
4. Wash every chamber with 500 μl sterile water.
5. Fill every chamber with 500 μl sterile PBS and store the coated slide for up to 7 days at 4°C.

   Take care that you do not damage the surface of the slide with the pipet tip during the coating process.

Prepare the exposure solutions and the endpoint-specific control solutions

6. Prepare a dilution series of the test compound stock solution in proliferation medium (for plating experiments under proliferating conditions; endpoint proliferation) or differentiation medium (for plating experiments under differentiating conditions; endpoints: migration, differentiation, or apoptosis).

   The final concentration of the vehicle should be the same in all dilutions and not surpass 0.25% (v/v) for DMSO or other organic solvents and 1% (v/v) for distilled water or PBS.

Table 12.21.1 Material Needed to Prepare the Endpoint-Specific Control Solutions

Assay conditions	Endpoint	Material
Proliferating	Proliferation by diameter increase	Proliferation medium without growth factors
	Proliferation by BrdU incorporation	Proliferation medium without growth factors
	Viability (Alamar Blue Assay)	Proliferation medium (at the end of the assay Triton X solution will be added)
	Cytotoxicity (Lactate Dehydrogenase assay; LDH assay)	Proliferation medium (at the end of the assay Triton X solution will be added)
Differentiating	Differentiation to neurons	Differentiation medium with 20 ng/ml EGF
	Differentiation to astrocytes	Differentiation medium with 20 ng/ml EGF
	Differentiation to oligodendrocytes	Differentiation medium with 50 ng/ml FGF-2
	Migration	Differentiation medium with 10 μM PP2
	Viability (Alamar Blue Assay)	Differentiation medium (at the end of the assay Triton X solution will be added)
	Cytotoxicity (LDH assay)	Differentiation medium (at the end of the assay Triton X solution will be added)
	Apoptosis (Live Dead assay)	Differentiation medium (16 hr prior to the end of the experiment, Staurosporine will be added)

7. Prepare a solvent control solution adding the same solvent used to deliver the compound to the medium at the same final concentration.
8. To calculate the effective concentration 50 (EC_{50}; concentration which produces a 50% decrease of the control values) prepare between five and seven seriated dilutions of the test compound with a dilution factor between 2 and 5. To calculate EC_{50} values, see last step of Basic Protocol 3.
9. For each serial dilution solution, prepare a volume considering that 100 μl of medium per sphere will be needed during the exposure period.
10. Fill the chambers of a coated 8-chamber slide with 500 μl of the exposure solutions for culturing neurospheres under differentiating conditions or the wells of a round-bottom 96-well plate with 100 μl of exposure solutions for culturing the neurospheres under proliferating conditions (Fig. 12.21.2).

    Do not plate the spheres in the outer wells of the 96-well plate; instead fill them with sterile water to avoid evaporation in the inner wells of the plate.

11. Depending on the endpoint to evaluate, prepare the endpoint-specific control solutions following the instructions in Table 12.21.1.

    We recommend plating the endpoint-specific controls following the order shown in Figure 12.21.2.

For the viability (Alamar Blue assay) and cytotoxicity assay (Lactate dehydrogenase assay; LDH assay) endpoint-specific controls, prepare only differentiation or proliferation medium without adding any reagent. Triton X solution will be added to these wells at the end of the assay. The same applies for the endpoint-specific control for the apoptosis assay (Live Dead Assay). Here, staurosporine will be added 16 hr prior to the end of the assay (see Basic Protocol 3).

12. Prepare a background control for the endpoints based on fluorescence or luminescence assays as follows:

    Viability (Alamar Blue Assay): differentiation or proliferation medium
    Cytotoxicity (LDH assay): differentiation or proliferation medium
    BrdU incorporation: proliferation medium.

    It is important to remark that in the background control wells or chambers, no spheres will be plated.

13. Add the necessary control solutions to the coated 8-chamber slide (for differentiation conditions) or to the 96-well plate (for proliferation conditions).

    If culturing the spheres longer than 72 hr, prepare an extra volume of all needed solutions to replace half of the culture medium every 2 to 3 days.

Sort and plate neurospheres manually

14a. Fill a 6-cm petri dish with either proliferation medium (for plating experiments under proliferating conditions) or differentiation medium (for plating experiments under differentiating conditions).

15a. Take a 10-cm petri dish with chopped rat or human neurospheres (see Basic Protocol 1) and sort neurospheres of ~0.3 mm in diameter with a 100-µl tip under a binocular microscope using a micrometer.

16a. Pipet the sorted neurospheres into the 6-cm petri dish and check if they are uniform in size and shape.

17a. For experiments under proliferating conditions, transfer one sphere to a 96-well filled with the experimental solutions in less than 10 µl medium with a 100-µl tip. For experiments under differentiating conditions, transfer five spheres to a filled chamber of a coated 8-chamber slide in 20 µl medium with a 100-µl tip and position them as shown in Figure 12.21.2.

18a. Incubate the 8-chamber slide or the 96-well plate with the spheres at 37°C and 5% CO_2 for the desired culture period, depending on the endpoint of study.

    Take pictures of each sphere before placing the 96-well plate in the incubator for later use in proliferation experiments

Sort and plate neurospheres with the COPAS Large Particle Sorter

14b. Turn on the computer and the instrument, check the tanks and, if necessary, refill sterile PBS and empty the waste.

15b. Start the Biosort program and follow the program instructions:

    i. Turn on the pressure and the laser.
    ii. Check the pressure: sheath pressure is ideally 4 and should be constant during the measurement, sample pressure should be between 0.15 and 0.5; if so, press "pressure ok".

    Priming of the instrument starts automatically.

16b. Rinse the instrument with ethanol and sterile water (therefore, only turn on the sample valve).

17b. Fill the chopped neurospheres of one petri dish (depending on how many neurospheres you would like to plate) into the sample chamber.

18b. Choose the desired plate format for sorting under "plate format."

19b. Delay = time between measurement and sorting (is standard 11 msec).

20b. Width = droplet size (should be 5 for small neurospheres, which is equal to 1.8 μl per droplet).

21b. Choose the number of events per well.

For sorting into a 96-well plate, choose "one event" per well; for sorting into an 8-chamber slide, choose "five events" per well.

22b. Set threshold: signal = 20 and TOF = 50.

23b. Check the positioning with the lid of the plate by choosing "Align Plate Handler and Stage" under "Tools."

i. Click "Well A1" and choose "test drop."
ii. Check if the drop is positioned correctly, if not, correct with arrow keys.
iii. Click "Last Well" and choose "test drop."
iv. Check if the drop is positioned correctly, if not, correct with arrow keys.
v. Click "Done" and save settings.

24b. Click "Load Plate."

96-well plate/8-chamber slide moves to the starting position.

25b. Click "Fill Plate."

Neurospheres are sorted into wells/chambers.

26b. After the sorting process is finished, clean the device properly as follows:

i. Empty the sample chamber and the recovery chamber (neurospheres that were not sorted can be kept in culture later).
ii. Fill the sample chamber with 10 to 15 ml cleaning solution and click "sample valve" until the solution passes through.
iii. Remove tick, fill with sterile water and click "sample valve" until almost everything passes through (take care that the device never runs empty).
iv. Click "Clean" to remove air bubbles.
v. Unscrew the small PBS bottle to ventilate the device.
vi. Empty the waste and recovery chamber.

27b. Close the software first, and then turn off the device, the laser, and the pump.

28b. In case of a 96-well plate, the sorting and plating process is finished here; in case of an 8-chamber slide, the five spheres per chamber still need to be positioned manually as shown in Figure 12.21.2.

29b. Incubate the 8-chamber slide or the 96-well plate with the spheres at 37°C and 5% CO_2 for the desired culture period depending on the endpoint of study.

Take pictures of each sphere before placing the 96-well plate in the incubator for later use in proliferation experiments.

INVESTIGATION OF DEVELOPMENTAL NEUROTOXICITY ENDPOINTS UNDER PROLIFERATING AND DIFFERENTIATING CONDITIONS

The "Neurosphere Assay" offers the possibility to evaluate several endpoints which represent relevant processes of neurodevelopment. During the assay, neurospheres can be cultured under proliferating conditions to later measure proliferation by means of diameter increase or by BrdU incorporation, or can be cultured under differentiating conditions to finally evaluate migration, differentiation to astrocytes, neurons, and oligodendrocytes, or apoptosis. Under both conditions, the viability/cytotoxicity of neurospheres is also evaluated to distinguish between general cytotoxicity and specific effects on each endpoint. In this protocol, the steps needed to evaluate proliferation, migration, differentiation, and apoptosis are described, as well as the supportive viability and cytotoxicity assays for both culturing conditions.

Materials

Pictures of rat or human proliferating neurospheres taken at day 0 of culture (from Basic Protocol 2)
96-well plate with rat or human neurospheres cultured under proliferating conditions (from Basic Protocol 2)
CytoTox-One Reagent (see recipe)
Triton X solution (see recipe)
CytoTox-One kit (Promega GmbH) containing Assay Buffer and Substrate Mix
CellTiter-Blue Reagent (CTB; Promega GmbH)
Proliferation medium without growth factors (see recipe)
BrdU cell proliferation ELISA kit (Roche Applied Sciences; chemiluminescent) containing:
- BrdU labeling reagent
- Anti-BrdU stock solution
- Antibody dilution solution
- FixDenat solution
- 10× washing solution
- Substrate component A solution
- Substrate component B solution.

BrdU labeling solution (see recipe)
Accutase
Anti-BrdU-POD working solution (see recipe)
Washing solution (see recipe)
Substrate solution (see recipe)
8-chamber slide with rat or human neurospheres cultured under differentiating conditions (from Basic Protocol 2)
Differentiation medium (see recipe)
12% (w/v) paraformaldehyde (PFA; see recipe)
1× PBS (see recipe)
PBS-T (see recipe)
Goat serum
Primary and secondary antibodies (see Table 12.21.2)
Nuclear stain (see Table 12.21.2)
Distilled water
Mounting medium: Aqua Poly/Mount (Polysciences Europe GmbH)
Staurosporine (Sigma-Aldrich)
Propidium iodide (1 mg/ml solution in water; Sigma-Aldrich)

ImageJ software
Incubator

Table 12.21.2 Antibody Dilutions Needed for Immunocytochemical Staining

	Dilution factor	Supplier
Primary antibodies		
Rabbit IgG anti-GFAP	1:100	Sigma-Aldrich
Mouse IgG anti-β(III)tubulin	1:100	Sigma-Aldrich
Mouse IgM anti-O4	1:200	R&D Systems
Secondary antibodies		
Alexa Fluor 488 anti-mouse IgG	1:250	Life Technologies GmbH
Alexa Fluor 546 anti-rabbit IgG	1:100	Life Technologies GmbH
Alexa Fluor 488 anti-mouse IgM	1:250	Life Technologies GmbH
Nuclear stain		
Hoechst 33258 (see recipe)	1:100	Sigma-Aldrich

Flat-bottom transparent 96-well plate
Fluorescence reader with excitation 530 to 570 nm and emission 580 to 620 nm filter pair
Water bath
Black 96-well plate
Multichannel pipet
Hairdryer
Paper towels
Luminescence reader
Phase-contrast microscope with camera
Parafilm
Coplin jar
Humidified box
Cotton sticks
Slide cover slips
Graph Pad Prism

Assessment of proliferation by diameter increase (proliferating conditions)

The proliferation assay is performed to determine the ability of NPC to divide and generate new cells over time. A simple way to assess proliferation is measuring the diameter increase of floating neurospheres. However, this method is not sensitive enough to detect subtle changes in proliferation after a few days of exposure; therefore, exposure times of at least 1 week are recommended. For shorter exposure times, a more sensitive alternative method is presented in step 1b, the assessment of proliferation by BrdU incorporation. To evaluate proliferation, you will need the pictures of the proliferating neurospheres taken at day 0.

1a. At the desired evaluation day, take a picture of each sphere cultured in a round-bottom 96-well plate. We recommend evaluating the diameter increase until day 7 (minimum and maximum) for rat neurospheres and until day 7 (minimum) or 14 (maximum) for human neurospheres. Additionally, it is useful to take pictures of the neurospheres every feeding day to monitor the diameter increase over the whole experimental period.

2a. Measure the diameter of each sphere twice in a perpendicular angle using ImageJ software and calculate the mean diameter of the neurospheres at each concentration (see Fig. 12.21.1).

3a. Plot the diameter increase (in μm) over time (in days) in a dot plot and calculate the slope of the line of best fit for each concentration.

During the proliferation period, remember to feed the neurospheres by replacing half of the medium every 2 to 3 days with fresh proliferation medium. It is recommended to move the neurospheres while feeding by pipetting with a 100-μl tip to avoid adhesion and differentiation.

Assess cytotoxicity by LDH assay (proliferating conditions)

LDH is a cytoplasmic enzyme that catalyzes the oxidation of lactate to pyruvate. The LDH assay determines the integrity of cell membrane of the cultured NPCs by detecting the presence of LDH in the culture medium, which is used as an indicator of cell death.

4a. Perform the cytotoxicity assay on the same day as the proliferation assessment by using the same neurospheres.

5a. Pre-warm the CytoTox-One Reagent at room temperature.

6a. Add 5 μl of Triton X solution per well to the endpoint-specific control wells.

7a. Incubate at standard conditions for 20 min.

8a. Transfer 50 μl of the culture medium of the exposed neurospheres from each well of the culturing plate to a new transparent flat-bottom 96-well plate.

Take care that you do not remove the neurosphere from its well during that process.

9a. The culturing plate with the neurospheres can be refilled with 50 μl of fresh medium and further incubated, or can be directly used to perform a viability assay as detailed in 13a to 18a.

10a. Add 50 μl CytoTox-One Reagent to each well of the new flat-bottom 96-well plate.

11a. Incubate for 4 hr at 37°C.

12a. Shake the plate by hand for 10 sec and measure fluorescence at 544/590 nm directly in the same 96-well plate.

The half-life of LDH in medium is ~9 hr at 37°C. But collected medium can be stored for 2 to 4 weeks at −20°C prior to adding the CytoTox-One Reagent without remarkable loss of LDH activity.

Assess viability by Alamar Blue Assay (proliferating conditions)

The Alamar Blue Assay measures the metabolic activity of the cultured cells by determining the activity of the mitochondrial respiratory chain. The substrate resazurin is converted by metabolic active cells into the fluorescent pigment resorufin. The degree of substrate conversion is directly correlated with the respiratory activity of the culture and used as an indicator of cell viability.

13a. Perform the viability assay at the same day as the proliferation assay by using the same neurospheres. If an LDH assay has been performed prior to the viability assay, the 96-well plate from step 9a continues to be processed as follows. If the LDH assay was not performed before, add 5 μl of Triton-X solution per well to the endpoint-specific control wells, incubate for 20 min under standard conditions, and proceed.

14a. Thaw CellTiter-Blue Reagent (CTB) in a 37°C water bath and protect it from direct light exposure.

15a. Dilute CTB 1:3 with proliferation medium without growth factors.

16a. Add the CTB dilution in a proportion of 1:4 to each well.

17a. Incubate using standard cell culture conditions for 2 hr.

18a. Shake the plate by hand for 10 sec and measure fluorescence at 544/590 nm directly in the same 96-well plate.

To analyze the results see "Data evaluation by determination of EC_{50} values" below.

Assess proliferation by BrdU incorporation (proliferating conditions)

Alternatively to the measurement of the diameter increase, a direct proliferation assessment can be performed by quantification of DNA synthesis. BrdU is a thymidine analogue, which incorporates into DNA of newly generated cells. Based on a chemoluminescent reaction, this assay uses an antibody against BrdU, which is conjugated to a peroxidase. In presence of H_2O_2 the peroxidase catalyzes the oxidation of luminol to oxyluminol and the emitted light is measured.

1b. Every day during the assay, move the neurospheres once by pipetting with a 100-µl tip to avoid adhesion and differentiation. This is especially important for rat neurospheres.

2b. At a time point 16 hr prior to the determination of BrdU incorporation, add 10 µl per well of BrdU labeling solution to each neurosphere cultured in the 96-well. We recommend evaluating BrdU incorporation on day 3 for both rat and human neurospheres. Do not add the BrdU labeling solution to the BrdU background control wells.

The 16 hr incubation period with the BrdU labeling solution can be shortened or prolonged. However, it should not be longer than 24 hr to avoid cytotoxicity.

3b. On the desired evaluation day, perform the viability and/or cytotoxicity assay as described in steps 5a to 19a.

4b. Put 25 µl of prewarmed Accutase per well into a black 96-well plate.

5b. Transfer every neurosphere from the culturing plate to a well of the black 96-well plate into the Accutase drop with a 100-µl tip using as little medium as possible.

6b. Incubate for 10 min at 37°C.

7b. Create a single-cell suspension by pipetting up and down with a 100-µl multichannel pipet at least 10 times.

8b. Remove the Accutase by heating the plate with a hairdryer.

9b. Add 200 µl/well of FixDenat solution to each well, and incubate for 30 min at room temperature to fixate the cells and denaturize the DNA.

10b. Remove the FixDenat solution by flicking off and tapping on a paper towel.

11b. Add 100 µl/well of Anti-BrdU-peroxidase (POD) working solution.

12b. Incubate for 1 hr at room temperature.

13b. Remove Anti-BrdU-peroxidase solution by flicking off and tapping on a paper towel.

14b. Rinse the wells three times with 200 µl/well of washing solution.

15b. Remove the washing solution by tapping on a paper towel.

16b. Add 100 µl/well of substrate solution and incubate for 5 min at room temperature.

17b. Measure luminescence with a luminometer.

To analyze the results see "Data evaluation by determination of EC_{50} values" below.

Assess migration (differentiating conditions)

The endpoint of migration reflects the ability of the NPCs to move from proliferating niches to their final position in brain. In this protocol we present a simple method to evaluate the ability of NPCs to migrate out of the sphere core by measuring the maximum covered distance over the coated surface in a given period of time.

1c. On the desired evaluation day, take a picture with a phase-contrast microscope of the whole migration area of each sphere cultured in an 8-chamber slide.

We recommend evaluating migration on day 3 for both rat and human neurospheres.

2c. Measure the four radii of the migration area of each neurosphere in perpendicular angles from the edge of the neurosphere to the furthest migrated cells using ImageJ and calculate the mean to obtain the migration distance of each neurosphere.

Assess cytotoxicity by LDH assay (differentiating conditions)

3c. Perform the cytotoxicity assay on the same day as migration or differentiation assessment by using the same neurospheres.

4c. Prewarm the CytoTox-One Reagent at room temperature.

5c. Add 10 µl of Triton X solution to the endpoint-specific control chamber.

6c. Incubate at standard conditions for 20 min.

7c. Fill two wells of a transparent flat-bottom 96-well plate, transferring 100 µl of culture medium of the exposed neurospheres into each well.

8c. The culturing slide with the neurospheres can be refilled with fresh medium and further incubated, or can be directly used to perform a viability assay as detailed in 12c to 18c.

9c. Add 100 µl of CytoTox-One Reagent to each well of the new flat-bottom 96-well plate.

10c. Incubate for 4 hr at 37°C.

11c. Shake the plate for 10 sec and measure fluorescence at 544/590 nm directly in the same 96-well plate.

Assess viability by Alamar Blue Assay (differentiating conditions)

12c. Perform the viability assay at the same day as the migration or differentiation assays by using the same neurospheres. If a cytotoxicity assay has been performed prior to the viability assay, the 8-chamber slide from step 11c continues to be used as follows. If the cytotoxicity test was not performed, add 10 µl of Triton X solution per chamber to the endpoint-specific control chamber, incubate for 20 min at standard conditions and proceed.

13c. Thaw CellTiter-Blue Reagent (CTB) in a 37°C water bath and protect it from direct light exposure.

14c. Dilute CTB 1:3 with differentiation medium.

15c. Add the CTB dilution in a proportion of 1:4 to each chamber.

16c. Incubate using standard cell culture conditions for 2 hr.

17c. Fill two wells of a transparent flat-bottom 96-well plate, transferring 100 µl of culture medium of the exposed neurospheres into each well.

18c. Shake the plate for 10 sec and measure fluorescence at 544/590 nm directly in the same 96-well plate.

Assess differentiation to neurons, astrocytes, and oligodendrocytes (differentiating conditions)

The term differentiation refers to the process of NPCs developing into the three major neural cell types: neurons, astrocytes, and oligodendrocytes. In this assay, neurospheres are fixed and the percentage of cells in the migration area, which are differentiated into each of these cell types is assessed by means of immunocytochemistry (ICC) using βIII-tubulin, GFAP and O4 antibodies, as well as Hoechst as a nuclear staining.

19c. Fix the cells by adding 12% PFA to each chamber to obtain a final concentration of 4% PFA and incubate for 30 min at 37°C.

20c. Remove the 4% PFA solution from each chamber and add 500 µl of 1× PBS. At this time point, the slides can be stored sealed with Parafilm at 4°C for maximum 4 weeks.

21c. Empty all wells and carefully take off the plastic gasket of the 8-chamber slide.

22c. Wash the slides two times for 5 min in a Coplin jar with ~90 ml 1× PBS.

23c. Prepare the primary antibodies following the dilutions recommended in Table 12.21.2 using PBS for surface epitopes (O4) or PBS-T for intracellular epitopes (βIII-tubulin and GFAP). Always add 10% goat serum.

24c. Place the slide in a humidified box, dry the edges of each area of the 8-chamber slide with a cotton stick carefully, and add 25 µl of primary antibody solution to each well area, ensuring that all neurospheres are covered by the solution. Incubate for 1 hr at 37°C.

25c. Wash the slide three times for 5 min in a Coplin jar in ~90 ml 1× PBS.

26c. Meanwhile, prepare the secondary antibodies solutions following the dilutions recommended in Table 12.21.2 with PBS supplemented with 1% Hoechst (nuclear stain) and 2% goat serum.

27c. Incubate the slide with the secondary antibody solution as described in 24c, but for 30 min at 37°C.

28c. Wash the slide three times for 5 min in a Coplin jar in ~90 ml 1× PBS.

29c. Dip the slide once in distilled water to avoid saline residuals.

30c. Carefully dry the edges of each well again with the cotton stick and add a drop of mounting medium to each well and place a cover slip onto the slide. Remove abundant mounting medium with a paper towel and gently press down.

31c. Let the slides dry at least overnight at 4°C and microscopically examine the slide by taking two fluorescent pictures per neurosphere at opposite sides of the migration area.

The slides can be stored in the dark at 4°C for up to a month before examination.

32c. To evaluate differentiation, count the total number of Hoechst-positive cell nuclei and the number of cells stained with GFAP, β-III tubulin, or O4 depending on the

cell population of study. Calculate the percentage of cells expressing the marker of interest from total number of nuclei.

To analyze the results, see "Data evaluation by determination of EC_{50} values" below.

Assess apoptosis by Live Dead Assay (differentiating conditions)

The Live Dead Assay is performed to determine the amount of apoptotic cells. It is based on the principle of nuclear condensation in apoptotic cells, which is visualized by staining the cells with Hoechst and propidium iodide. Hoechst enters every cell and intercalates into the DNA while propidium iodide only enters cells with disturbed membrane integrity and intercalates into the DNA. By adding both dyes to living cells, all cell nuclei are stained by Hoechst, whereas only late apoptotic condensed cell nuclei are also stained by propidium iodide. Condensed and apoptotic nuclei are then double stained for Hoechst and propidium iodide and are counted as apoptotic cells.

1d. At a time point 16 hr prior to the end of the experiment, add Staurosporine to the endpoint-specific control chamber to obtain a final concentration of 0.5 μM.

2d. Add 1 μl propidium iodide and 5 μl Hoechst (nuclear stain) to every chamber of the 8-chamber slide 30 min prior to the end of experiment and incubate for 30 min using standard conditions.

3d. Fix the neurospheres by adding 250 μl of 12% PFA to each chamber for 30 min at 37°C.

4d. Remove the solution from the wells, remove the chambers, wash the slide in a Coplin jar with ~90 ml distilled water, embed with 8 drops of mounting medium, and keep the slide overnight at 4°C for drying.

5d. Take two fluorescence pictures of the Hoechst-propidium iodide co-staining per neurosphere at opposite sides of the migration area.

6d. For evaluation, count the total number of Hoechst-positive cell nuclei, as well as the number of the condensed double positive nuclei (Hoechst-propidium iodide). Calculate the percentage of double-positive nuclei from total number of nuclei to obtain the percentage of apoptotic cells.

Evaluate the data by determination of EC_{50} values

EC_{50} values need to be determined for every evaluated endpoint in both species by applying a nonlinear sigmoidal dose response curve fit to the obtained data points. For that reason, the data points of every single experiment are expressed in percentage of the respective vehicle control. To gain statistical relevant results, every experiment needs to be repeated for at least three times, and the data points of the single experiments are averaged in the end. The curves of the means (M) + SE are then fitted to calculate the EC_{50} values (Fig. 12.21.3).

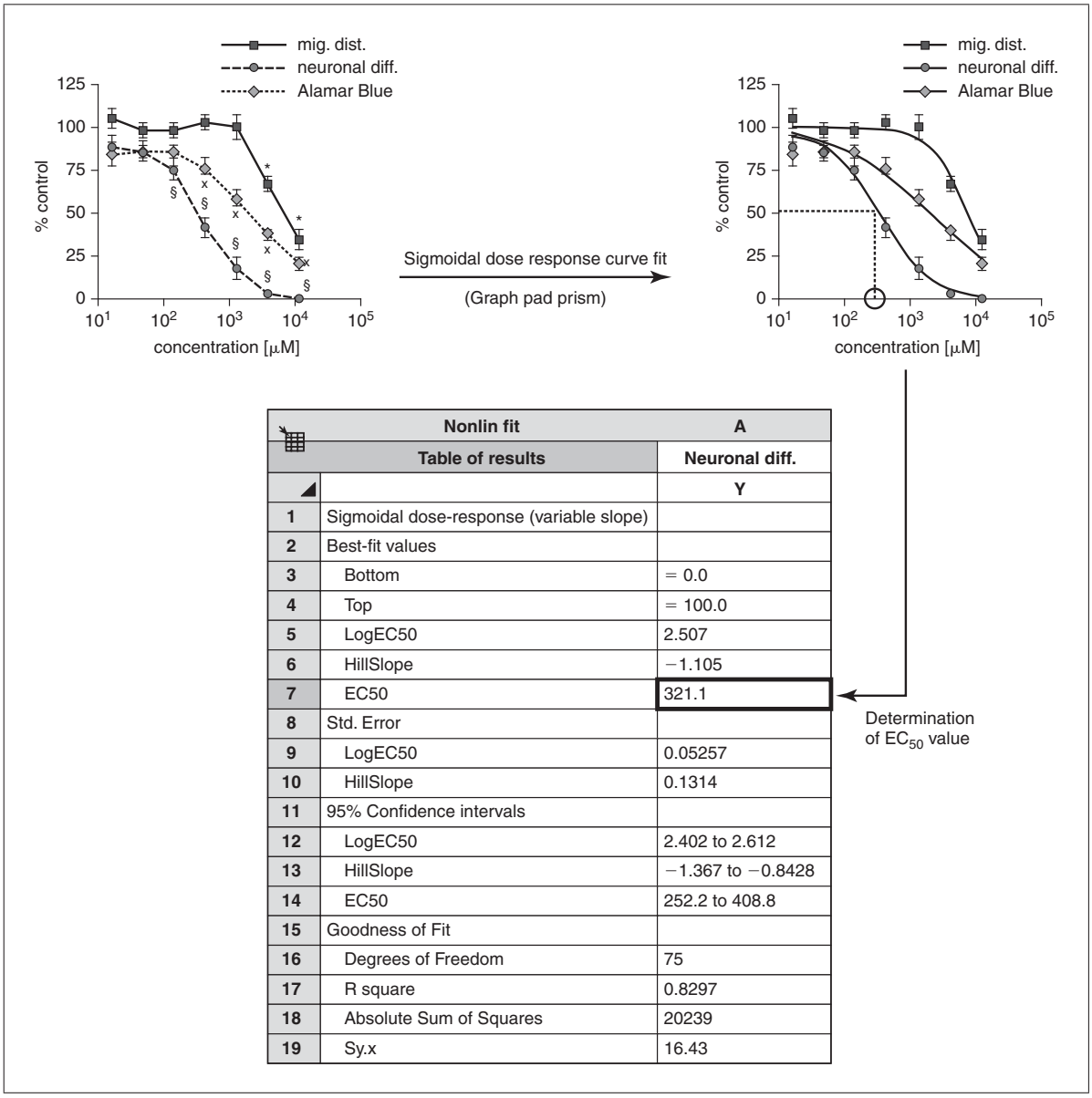
1e. For every endpoint: Express the data of every single experiment in percentage of the respective vehicle control and average the data of the single experiments to obtain a mean (M) + SE.

2e. Plot the data in a dot plot with a logarithmic *x*-axis to obtain a concentration response curve with Graph Pad Prism.

i. Transform the data to a logarithmic *x*-axis.
ii. Create a graph to visualize the concentration response curve.

3e. Apply a nonlinear sigmoidal dose response curve fit to the transformed data.

i. Set the Top value to 100 and the bottom value to 0.



Nonlin fit	A
Table of results	Neuronal diff.
	Y
1 Sigmoidal dose-response (variable slope)	
2 Best-fit values	
3 Bottom	= 0.0
4 Top	= 100.0
5 LogEC50	2.507
6 HillSlope	−1.105
7 EC50	321.1
8 Std. Error	
9 LogEC50	0.05257
10 HillSlope	0.1314
11 95% Confidence intervals	
12 LogEC50	2.402 to 2.612
13 HillSlope	−1.367 to −0.8428
14 EC50	252.2 to 408.8
15 Goodness of Fit	
16 Degrees of Freedom	75
17 R square	0.8297
18 Absolute Sum of Squares	20239
19 Sy.x	16.43

Figure 12.21.3 Determination of EC_{50} values. First, the results of the concentration-response experiments need to be expressed in percentage of the respective vehicle control and plotted with a logarithmic *x*-axis. Afterwards, a nonlinear sigmoidal dose response curve fit of the data needs to be performed to obtain a "table of results," from which the EC_{50} value can be extracted.

ii. Show the "results table" to obtain the calculated EC_{50} value, confidence intervals and the goodness of fit.
iii. Create a graph of the curve fit to check the fitting of the data points.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Anti-BrdU-POD working solution

Prepare a 1:100 dilution of the anti-BrdU stock solution (from the BrdU cell proliferation ELISA kit; Roche Applied Sciences) with the antibody dilution solution. Prepare immediately before use.

BrdU labeling solution

Prepare a 1:100 dilution of the BrdU labeling reagent (from the BrdU cell proliferation ELISA kit; Roche Applied Sciences) with proliferation medium without growth factors. Prepare immediately before use.

CytoTox-One Reagent

Add 11 ml of Assay Buffer to one vial of Substrate Mix. Gently mix to dissolve the substrate. Store for 6 to 8 weeks tightly capped and protected from direct light at −20°C.

Differentiation medium

DMEM (Gibco GlutaMAX, Life Technologies GmbH), Hams F12 (Gibco GlutaMAX, Life Technologies GmbH) 3:1 supplemented with 1% of N2 (Life Technologies GmbH) and antibiotic solution (100× penicillin/streptomycin) to 1× final. Store for 2 weeks at 4°C.

Hoechst 33258

Prepare a stock solution with a concentration of 10 mg/ml in dimethyl sulfoxide (DMSO; store for at least one year at −20°C) and dilute it 1:50 in distilled water to obtain a solution with a concentration of 0.2 mg/ml Store up to 6 months at 4°C.

Ovomucoid solution (for one brain)

Mix 975 µl DMEM with 10 µl Trypsin inhibitor (final concentration: 1 µg/ml, Sigma-Aldrich), 5 µl bovine serum albumin (final concentration: 500 µg/ml; Serva Electrophoresis GmbH) and 20 µl DNase I. Sterilize the solution with a 0.22-µm syringe filter. Prepare immediately before use.

PBS, 1×

Prepare a PBS 10× stock solution with 80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl. Adjust the pH to 7.5 with HCl and store at room temperature. Prepare a 1× working solution by dilution with distilled water and store up to 3 months at room temperature.

PBS-T

0.1% (v/v) Triton X-100 in PBS
Store up to 3 months at room temperature

Paraformaldehyde (PFA), 12%

Dissolve 12 g PFA in 100 ml phosphate-buffered saline (PBS; see recipe) and add 5 drops of 1 N NaOH. Heat the solution carefully to 70° to 80°C in a fume hood and cool to room temperature. Divide into 1-ml aliquots. Store aliquots up to 1 year at −80°C and use them freshly.

Poly-D-lysine

Dissolve poly-D-lysine (Sigma-Aldrich, cat. no. P0899) at 0.1 mg/ml in sterile water
Store up to 1 month at −20°C

Proliferation medium

DMEM (Gibco GlutaMAX, Life Technologies GmbH) and Hams F12 (Gibco GlutaMAX, Life Technologies GmbH) 3:1 supplemented with 2% B27 (Life Technologies

(*continued*)

GmbH). Epidermal growth factor (EGF, Life Technologies GmbH) and recombinant human fibroblast growth factor (FGF, R&D Systems) are dissolved at 10 μg/ml in sterile PBS containing 0.1% BSA and 1 mM DTT and stored at −20°C. EGF is diluted in medium at a final concentration of 20 ng/ml. FGF is diluted in medium at a final concentration of 20 ng/ml for human neurospheres and for rat neurospheres FGF is not added to the medium. Add antibiotic solution (100× penicillin/streptomycin) to 1× final. Store up to 2 weeks at 4°C.

Proliferation medium without growth factors

Same recipe as for proliferation medium (see recipe), but without adding EGF or FGF
Store up to 2 weeks at 4°C

Substrate solution

Prepare a 1:100 dilution of the substrate component B in the substrate component A solution (from the BrdU cell proliferation ELISA kit; Roche Applied Sciences). Prepare immediately before use.

Tissue digestion solution (for one brain)

Mix 970 μl MEM with 20 μl Papain (final concentration: 30 U/ml, Worthington Biochemical Corp) and pre-incubate the solution for 10 min at 37°C. After pre-incubation, add 10 μl DNase I (final concentration: 40 μg/ml, Worthington Biochemical Corp) to the Papain mixture. Sterilize the solution with a 0.22-μm syringe filter. Prepare immediately before use.

Triton X solution

9% (v/v) Triton X-100 in sterile water
Store up to 6 months at 4°C

Washing solution

Dilute the 10× washing solution (from the BrdU cell proliferation ELISA kit; Roche Applied Sciences) with distilled H_2O
Store for 1 week at 4°C

COMMENTARY

Background Information

In 1992, Reynolds, Weiss, and colleagues were the first ones generating neurospheres from brain tissue (Reynolds and Weiss, 1992; Reynolds et al., 1992). They isolated EGF-responsive cells from adult and embryonic mouse central nervous system (CNS) and cultured them under proliferating conditions forming clusters of undifferentiated cells. These clusters were called "neurospheres" and were dissociated to form secondary neurospheres (Reynolds and Rietze, 2005) or induced to differentiate into the three major CNS cell types: βIII-tubulin+ neurons, GFAP+ astrocytes and O4+ oligodendrocytes. Later, neurospheres were also generated from human embryonic and adult CNS (Svendsen et al., 1998; Carpenter et al., 1999; Kukekov et al., 1999).

Neurospheres from different species have the ability to mimic basic physiological processes of brain development like proliferation, migration, differentiation into neural effector cells, and apoptosis. Moreover, they maintain temporal and spatial correlation with in vivo brains concerning their potential to proliferate and differentiate (Zappone et al., 2000; Hitoshi et al., 2002; Ostenfeld et al., 2002; Parmar et al., 2002; Klein et al., 2005) and, as they are growing in 3-D structures, they express cell surface receptors and produce their own ECM to a larger extent than comparable 2-D cultures (Campos, 2004; Camarillo and Miranda, 2008; Elliott and Yuan, 2011). Due to these properties and their ability to mirror alterations in basic processes of neurodevelopment, in 2005 human neurospheres were first applied to study the effects of toxic

compounds on neurodevelopmental processes (Fritsche et al., 2005) and have been used as an in vitro model for evaluating hazards for brain development since (Moors et al., 2007, 2009; Gassmann et al., 2010; Schreiber et al., 2010; Fritsche et al., 2011).

Specific DNT testing is necessary to evaluate the impact of compounds on brain development because molecular initiating and key events specific for DNT differ to some extent from adult neurotoxicity (Bal-Price et al., 2013). Therefore, a variety of methods employing murine, rat, or human cell systems representing one or more neurodevelopmental processes in vitro have been established and utilized for DNT testing over the last decade (reviewed in Bal-Price et al., 2012). One advantage of models covering different species, as presented in this unit, is the possibility to compare species-specific toxicity pathways. Such information on species-specific toxicodynamics of compounds can be used to improve risk assessment by replacing the random toxicodynamic extrapolation factor from animals to humans by an experimentally determined number. That human cell models are recommended as most relevant to obtain predictive information on human toxicity was already stated within the novel document "Toxicity testing in the 21st century" (National Research Council, 2007). However, the diverse cellular systems currently available for DNT testing need to be characterized for their application domains. For one, it needs to be clearly defined which system is able to cover which functional DNT endpoint, e.g., NPC proliferation, neurite outgrowth, differentiation to neurons, astrocytes and oligodendrocytes, apoptosis, synaptogenesis, and network formation/activity (reviewed in Breier et al., 2010, Bal-Price et al., 2012). Secondly, presence of pathways relating to DNT-relevant key events in the respective cell models has to be ensured even to make detection of adversity possible. In the end, it has to be evaluated for each model to what extent the system resembles normal human physiology.

Within this context, the in vitro model presented in this unit fits in the general approach followed by the field of alternatives to animal testing: using human 3-D cellular systems to study physiologically relevant effects of toxic compounds. Moreover, it investigates species differences between humans and rodents for potentially improving DNT risk assessment. More specific pathway analyses are on the way to scientifically validate the method with regard to neurodevelopmental processes in vivo.

Critical Parameters and Troubleshooting

For investigating developmental neurotoxicity in vitro, it is advantageous to use primary three-dimensional organotypic co-culture cell systems, which are able to resemble basic processes of in vivo brain development. These requirements are fulfilled in NPCs grown as neurospheres, as they are able to proliferate, differentiate into neural effector cells, and undergo apoptosis (Moors et al., 2009). Moreover, as neurospheres can be obtained from humans and rodents, species differences can be investigated in complementary cellular systems, which is helpful for toxicological risk assessment. Furthermore, different endpoints of neurodevelopment can be investigated in parallel by using the same neurospheres. This approach is more efficient and reliable than single endpoint determinations.

Potential problems users may encounter with the protocols described in this manuscript might lie in the neurosphere culture, the choice of neurospheres for the experiments, and subsequent plating processes. The experimenter should make sure that spheres are fed on a regular basis. Neurospheres sorted for experiments should be round, should have a light color in the outer region and a darker color in the inner region. Novice experimenters may have the problem of irregular migration areas or neurospheres, which do not attach to the surface in experiments under differentiating conditions. This is usually due to damage to the coating, which may occur during the positioning of the neurospheres in the chamber. Therefore, scratching the coated surface with the pipet tip needs to be avoided. Possible problems users may encounter while performing the assays and methods described in Basic Protocol 3 are usually general to the overarching techniques, and are therefore beyond the scope of this protocol. For problems occurring while performing the Alamar Blue Assay, CytoTox One Assay and BrdU Assay, please refer to the Technical Bulletins of the respective manufacturer, and for further details on immunocytochemistry, please refer to Hoffman et al. (2008).

Anticipated Results

A critical parameter for the quality of the obtained results is the general performance of the vehicle controls and the endpoint-specific assay controls to assess the validity of each

performed experiment. For proliferation, human control neurospheres should increase ~200 μm or more in diameter over 7 days, whereas for rat control neurospheres a diameter increase of 100 to 150 μm within the same time period is usual. Measuring proliferation by BrdU incorporation results in relative luminescence units (RLUs). RLUs of the control spheres strongly depend on neurosphere size. However, for both species, RLUs of the endpoint-specific controls, which are neurospheres cultured without growth factors, should not be more than 30% to 40% of the vehicle control values. For the endpoint migration, a migration distance of 1000 to 1200 μm of human control NPCs within 3 days is anticipated, whereas human neurospheres treated with the src kinase inhibitor PP2 (Moors et al., 2007) should not migrate further than 60% to 80% of this value. For rat neurospheres, typical migration distances lie between 500 to 700 μm within 3 days and should not be lower than 500 μm. The PP2 control usually produces an inhibition of migration from 30% to 60% of the vehicle control. While migrating, human neurospheres generate ~10% neurons within all migrated cells after these three days in the migration area, whereas rat neurospheres typically generate 15% to 25% neurons. However, for human neurospheres, at least 5% neurons, and for rat neurospheres, at least 10% neurons should be formed to count the experiment as valid. The endpoint-specific control EGF should inhibit the differentiation into neurons to a value of at most 20% to 30% of the vehicle control to show a clear inhibition of differentiation (Ayuso-Sacido et al., 2010). Concerning the differentiation into oligodendrocytes, both, human and rat neurospheres should generate ~3% to 5% oligodendrocytes of all migrated cells, and FGF-2 should reduce this value to at least 20% (rat neurospheres) to 50% (human neurospheres) of the vehicle controls.

The determination of EC_{50} values for the endpoint proliferation, migration and differentiation as a method to compare the effects on the different endpoints within one species or between the two species requires compounds that have a negative impact on the abovementioned endpoints. However, for compounds that increase proliferation, migration, or differentiation, EC_{50} values cannot be calculated. But it has to be remarked that not only a negative but also a positive impact on our assay endpoints can indicate an abnormal neurodevelopment. During nervous system development, the biological processes pictured by the "Neurosphere Assay," which are proliferation, migration, differentiation, and apoptosis, occur simultaneously and in a coordinated manner. When only one of these processes is altered, e.g., by a toxic compound, nervous system development lacks perfection. Therefore, a compound altering a single endpoint of the assay at concentrations relevant for in vivo exposure is considered a DNT compound.

Time Considerations

Basic Protocol 1: Preparation of rat neurospheres requires half a day and provides enough material to work during one month.

Basic Protocol 2: For the neurospheres manual plating step, a trained experimenter needs only 10 min per plate or slide.

Basic Protocol 3: You need 3 days to complete the whole evaluation of the "Neurosphere Assay." All proliferation endpoints can be measured in half a day, and the differentiation ones in 2.5 days: 1 day until fixation of the slides and half a day for staining, plus 1 day more for picture acquisition and evaluation. A trained experimenter can evaluate three plates or four 8-chamber slides at the same time.

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Comparative Human and Rat "Neurosphere Assay" for Developmental Neurotoxicity Testing

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2.2. Automated neurosphere sorting and plating by the COPAS large particle sorter is a suitable method for high-throughput 3D *in vitro* applications

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*gleichberechtigte Erstautorenschaft

Toxicology *In vitro* [zur Publikation angenommen am 24. April 2012]

Die zur Verfügung stehenden Richtlinien für die toxikologische Testung auf Entwicklungsneurotoxizität sehen die Verwendung von Nagern vor. Solche Tierversuchsstudien sind jedoch aus ethischen Gründen fragwürdig und außerdem sehr zeit- und kostenintensiv. Aus diesem Grund besteht international die Übereinkunft darüber, dass die Entwicklung prädiktiver *in vitro* Methoden für eine effiziente Testung von Chemikalien sowie für eine Reduktion des Tierverbrauchs notwendig ist. Ein Ansatz unter eine Reihe von neuen Testmethoden für die Entwicklungsneurotoxizitätstestung verwendet Neurosphären, die dreidimensional kultivierte Zellaggregate normaler primärer neuraler Progenitorzellen darstellen. Da das Sortieren und Ausplattieren der Neurosphären einer der aufwendigsten Arbeitsschritte dieser Testmethode ist und damit eine effizienten Testung einer Vielzahl von Chemikalien in kurzer Zeit erschwert, war es das Ziel dieser Studie, zu analysieren, ob sich ein Sortierinstrument für größere Objekte (COPAS PLUS^{TM}, Union Biometric Inc.) für die automatische Sortierung und Plattierung von Neurosphären eignet. Dafür wurden manuell und automatisch sortierte und ausplattierte Neurosphären von Mensch, Maus und Ratte hinsichtlich ihrer Viabilität, ihrer Fähigkeit zu proliferieren, zu migrieren und zu differenzieren sowie ihres intrazellulären oxidativen Stresses miteinander verglichen. Unsere Ergebnisse zeigen, dass das COPAS Instrument in der Lage war, Neurosphären des gleichen Durchmessers zu sortieren und in 96-well Platten auszuplattieren. Dabei beeinflusste der automatische Sortier- und Plattierprozess weder Zellviabilität noch einen der funktionalen Endpunkte des Neurosphärenassays (Proliferation, Migration und Differenzierung zu Neuronen) im Vergleich zu manuell sortierten und ausplattierten Neurosphären. Darüber hinaus führte der automatische Sortier- und Plattiervorgang auch nicht zu einer erhöhten Generierung reaktiver Sauerstoffspezies. Aus diesem Grund hat sich das COPAS Instrument als ein geeignetes und nützliches Hilfsmittel für eine Erhöhung des Durchsatzes in der Neurosphärenforschung in den Bereichen Toxikologie, Neuroregeneration, Gehirnentwicklung, Wirkstoffforschung und Gehirnalterungsforschung erwiesen.

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Automated neurosphere sorting and plating by the COPAS large particle sorter is a suitable method for high-throughput 3D *in vitro* applications

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ABSTRACT

Existing guidelines for testing developmental neurotoxicity (DNT) propose investigations in rodents, which are ethically questionable as well as time and cost intensive. Thus, there is international agreement that predictive *in vitro* methods are needed to increase efficiency of testing and limit the number of animals used. One of a variety of novel approaches for DNT testing utilizes neurospheres, three-dimensional aggregate cultures of primary normal neural progenitor cells (NPCs). Because sorting and plating of single neurospheres is one of the most time-consuming steps within the assay, the aim of this study was to evaluate if the complex object parametric analyzer and sorter (COPAS PLUS™, Union Biometrica Inc.) is a suitable tool for automated sorting and plating of neurospheres. The results of the comparison of NPC viability, proliferation, migration, differentiation and intracellular oxidative stress between manually and COPAS sorted and plated neurospheres of different species show that the automation by the COPAS instrument does not influence the basic performance of neurospheres. Therefore, we consider the COPAS instrument as a useful tool for higher throughput neurosphere research in toxicology, neuroregeneration, brain development, drug development and brain aging research.

1. Introduction

A large number of chemicals are currently in use worldwide for which toxicity data is incomplete and/or lacking. As concern for human health is rising, there is the need for testing methods helping to predict toxicity of such compounds. This is especially true for the potential of chemicals to cause developmental neurotoxicity, as there are only five substances with a scientific basis sufficient to ascribe the potential to disturb human brain development (Grandjean and Landrigan, 2006; US EPA's Office of Pollution Prevention and Toxics, 1998). Guidelines for testing developmental neurotoxicity (DNT) include the US EPA test Guideline 870.6300 and the OECD-guideline 426, which proposes investigations in rodents, mainly rats. Such a DNT *in vivo* testing protocol requires the use of 140 dams and 1000 pups and is therefore ethically questionable and extremely time and cost intensive. Thus, for the need of increasing efficiency of testing (Andersen and Krewski, 2009; Kavlock et al., 2009) and at the same time limiting the number of animals used for such testing (Balls, 2009; Goldberg, 2002) world wide effort is arising to replace DNT animal experiments with predictive *in vitro* methods (Crofton et al., 2011).

To increase predictability, such *in vitro* alternative methods should ideally fulfill certain prerequisites: cells ought to be of human origin (National Research Council, 2007), non-immortalized and not derived from a tumor as this changes the normal cell physiology (Geerts et al., 2003; Moors et al., 2009), and reside in a three dimensional (3D) context (Yamada and Cukierman, 2007).Thereby, Yamada and Cukierman (2007) impressively summarize that 'Three-dimensional (3D) *in vitro* models span the gap between two-dimensional cell cultures and whole-animal systems'. As the key strengths of 3D cultures they indicate that (i) cell morphology and signaling are often more physiological than routine 2D cell culture, (ii) they permit rapid experimental manipulations and testing of hypotheses and (iii) they permit much better real-time and/or fixed imaging by microscopy than in animals. Nevertheless, also

Abbreviations: DNT, developmental neurotoxicity; NPC, neural progenitor cell; COPAS, complex object parametric analyzer and sorter; HTS, high throughput screening; ROS, reactive oxygen species; DCF, dichlorofluorescin; PDL, poly-D-lysin; AU, arbitrary units; TOF, time of flight; LDH, lactate dehydrogenase; TCHQ, tetrachlorohydrochinone; HBSS, Hanks' buffered saline solution; EXT, extinction; RLU, relative luminescence units.

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practicability has to be taken into consideration as for screening purposes cultures also have to be suitable for medium to high throughput screening (HTS) approaches.

During the last years we have established and characterized an innovative cell system for DNT testing consisting of primary normal neural progenitor cells (NPCs). These cells grow as 3D neurospheres in culture and are widely used as model systems for neurogenesis and neural development (rev. in Jensen and Parmar, 2006). For species comparisons neurospheres are also prepared from rodent mouse and rat pups. We showed that these 3D cultures mimic fundamental processes of brain development in culture, like NPC proliferation, migration, differentiation and apoptosis. Moreover, this model allows studying the effects of putative developmental neurotoxins not only on a functional, but also on a molecular level (Fritsche et al., 2005, 2011; Gassmann et al., 2010; Moors et al., 2007, 2009; Schreiber et al., 2010). So far we have mainly focused on characterization of this unique cell system as well as on identification of toxicity pathways in a low throughput for single-picked compounds by employing 'The neurosphere assay' (Fig. 1 in Breier et al., 2010; Fritsche et al., 2011). To be applicable for testing needs, it is crucial to improve the testing capacities to a medium-throughput system. Because sorting and plating of single neurospheres into multi-well plates is one of the most time-consuming steps within the neurosphere assay the aim of this study was to evaluate if the large particle flow cytometer COPAS PLUS (Union Biometrica Inc.) is a suitable tool for automated sorting and plating of neurospheres. Therefore, this technique is not only useful for *in vitro* testing of developmental neurotoxicity but also for other neurosphere applications in the fields of neuroregeneration, brain development, drug development and brain aging research.

For the establishment of such automation it is of highest importance that the automation process itself has no influence on the general cell functions and measured endpoints. Therefore, we compared cell viability, proliferation, migration and differentiation of manually to COPAS sorted and plated neurospheres of different species. Additionally, we determined levels of intracellular reactive oxygen species (ROS) by a dichlorofluorescin (DCF)-assay as a mea-

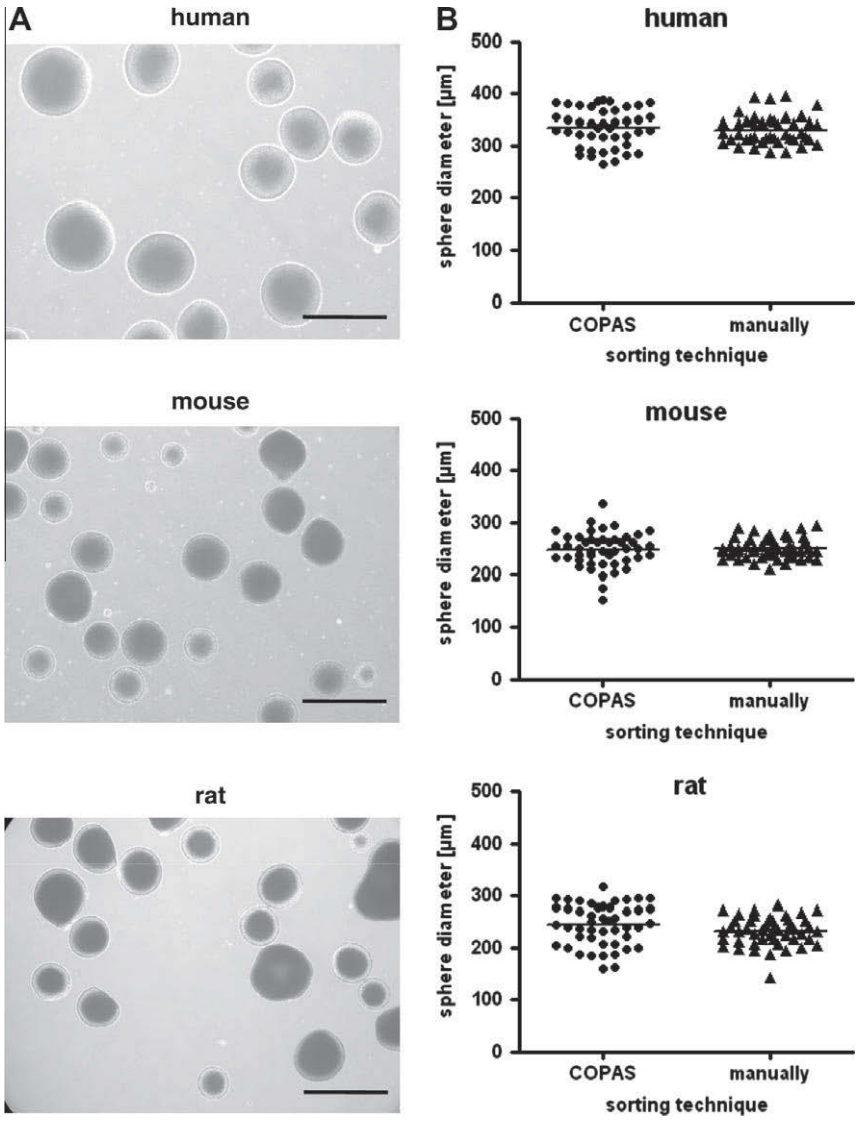

Fig. 1. Assessment of sorting accuracy. Microscopic images of the human, mouse and rat neurosphere culture before sorting. Scale bar = 500 µm (A). Forty to 50 neurospheres were sorted in a 96-well plate by the COPAS instrument (standard settings) and manually under a microscope . The diameter was analyzed with the metamorph program (Molecular Devices Corporation). One representative experiment/species is shown (B).

sure for cellular stress possibly arising from the sorting and plating process. The results show that the automation of plating and sorting by the COPAS instrument does not influence the basic performance of neurospheres and illustrate the usefulness of this instrument for higher throughput neurosphere research.

2. Materials and methods

2.1. Cell culture

Normal human neural progenitor cells used in this study were purchased from Lonza Verviers SPRL (Verviers, Belgium).

For rat neurosphere cultures, brains of wild-type Wistar rats (Charles River Laboratories International, Wilmington, MA, USA) were removed at postnatal day 5 and NPCs of mouse origin were prepared from postnatal day 3 C57/BL6 mice pups (Charles River Laboratories International, Wilmington, MA, USA). Heads were placed into phosphate-buffered saline and brains were dissected, transferred to minimal essential medium (MEM) and mechanically dissociated. Papain/DNAse solution (Worthington Biochemical Corporation, Lakewood, NJ) was added, and the suspension was incubated for 30 min at 37 °C in a humidified atmosphere. Afterward, the tissue suspension was triturated to obtain a single-cell suspension and a trypsin inhibitor solution was added to stop the tissue digestion. The cell suspension was centrifuged at 800 rpm for 5 min and the pellets were resuspended and plated in 10-cm petridishes. Neurospheres then form spontaneously by cell proliferation and re-aggregation. The animals were maintained in an accredited on-site testing facility according to the guidelines provided by the Society for Laboratory Animals Science (GV-SOLAS). They were treated humanely and with regard for alleviation of suffering.

Human and rodent neurospheres were cultured in proliferation medium [DMEM and Hams F12 (3:1) supplemented with B27 (Invitrogen GmBH, Karlsruhe, Germany), 20 ng/mL epidermal growth factor (EGF; Biosource, Karlsruhe, Germany), 100 U/mL penicillin, and 100 μg/mL streptomycin] in a humidified 92.5% air/7.5% CO_2 incubator at 37 °C in suspension culture.

Differentiation was initiated by growth factor withdrawal in differentiation medium [DMEM and Hams F12 (3:1) supplemented with N2 (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin] and plating onto poly-D-lysine (PDL)/laminin-coated chamber slides.

2.2. Flow cytometry using COPAS PLUS for analysis and dispensing of neurospheres

Neurospheres were sorted manually under a binocular microscope and plated with a 100 μL pipette or the sorting was performed with a complex object parametric analyzer and sorter (COPAS) instrument (Union Biometrica, Holliston, MA). Neurospheres were analyzed and dispensed by COPAS sorting by pulse shape analysis (Profiler II) which digitizes the object resulting in an optical profile of each object (signals generated by a 488 nm solid state laser). Profiler II will graphically show the location and intensity of the optical parameters and allows for extended sorting abilities with user definable sort criteria for profile peak heights, widths, locations and number for each optical parameter. Pulse shape diagram recording was triggered by the opacity signal [threshold > 20 AU (arbitrary units); signal gain factor 1; measuring range 0–65,500 AU]. Neurosphere size is expressed as time-of-flight [(TOF); gated range was set so to exclude all debris (230–1850 TOF, arbitrary units]. Peak profiling was initiated at >20,000 AU; width over threshold 100–600 AU. The COPAS-device was operated at a frequency of 2.5 MHz and coincidence settings for dispensing into microtiter plates were adjusted to 'enhanced'-mode, which allows other selected neurospheres to coincide within the same droplet.

The following instrument settings were used: delay 13, width 5.5 and sheath fluid pressure 3.9–4.1. The sample concentration fluid pressure was set to maintain an analysis or acquisition sort frequency of 1–2 events/s. The mixer speed was set on 50% and COPAS sorted neurospheres were plated directly into 96-well plates (proliferating conditions) or coated chamber-slides (differentiating conditions). After the plating the neurospheres were photographed and the neurosphere diameters were assessed with the Metamorph analysis software package (version 7.1.7.0; Universal Imaging Corp., West Chester, PA, USA).

2.3. Cell viability and cytotoxicity assays

For the cytotoxicity measurement after sorting the lactate dehydrogenase (LDH) assay (CytoTox-One; Promega, Mannheim, Germany) was used. It assesses cell death by measuring LDH that leaks out of dead cells into the media. We performed the assay according to the manufacturer's instructions. Briefly, supernatants of sorted neurospheres were collected and incubated with an equal amount of CytoTox-One reagent for 4 h before the detection of fluorescence (excitation, 540 nm; emission, 590 nm). Additionally cell viability was measured using the Alamar Blue assay (CellTiter-Blue assay Promega) as previously described (Moors et al., 2007). The assay is based on measurements of the mitochondrial reductase activity by conversion of the substrate resazurin to the fluorescent product resorufin by mitochondrial reductases, which can be assessed in a fluorometer (excitation, 540 nm; emission, 590 nm). In both assays cells completely lysed by 0.36% Triton X-100 (Sigma–Aldrich) served as toxicity control. As background control wells with the respective medium but without cells were used.

2.4. Proliferation analysis

For quantification of NPC proliferation the Cell Proliferation ELISA, BrdU (chemiluminescent) from Roche (Mannheim, Germany) was used according to the manufacturer's instructions with a BrdU incubation period of 16 h. The technique is based on the incorporation of the pyrimidine analogue BrdU (instead of thymidine) into the DNA of proliferating cells. After its incorporation into DNA, BrdU is detected by an immunoassay. For such analyses, adherent cell monolayers are needed, which are achieved by incubation of neurospheres for 10 min at 37 °C in accutase (AccuMax PAA, Pasching, Austria) in a 96 well plate after the 16 h BrdU incubation time and subsequent singularization by pipetting with a 100 μl tip. To get rid of the accutase and fix the cells on the plate bottom the liquid was completely removed by heating the plate with a hairdryer. Spheres cultivated in proliferation medium without EGF served as negative control and for correction of unspecific binding of the BrdU antibody some spheres were cultured without BrdU. Plates were then read in a luminometer (Tecan, Crailsheim, Germany).

2.5. Migration analysis and differentiation analysis

Migration distances were assessed 48 h after initiation of cell wandering by taking photographs of migrated spheres and measuring the distances from the residing sphere core to the furthest migrated cells at four pre-defined different positions. Thereby, four measurements of five spheres/condition result in one experimental *n*. Another 24 h later, after a total time of 72 h differentiation analyses were performed as previously described (Moors et al., 2007, 2009). Briefly, differentiated spheres were fixed in 2% paraformaldehyde for 30 min at 37 °C and neurons were then identified by

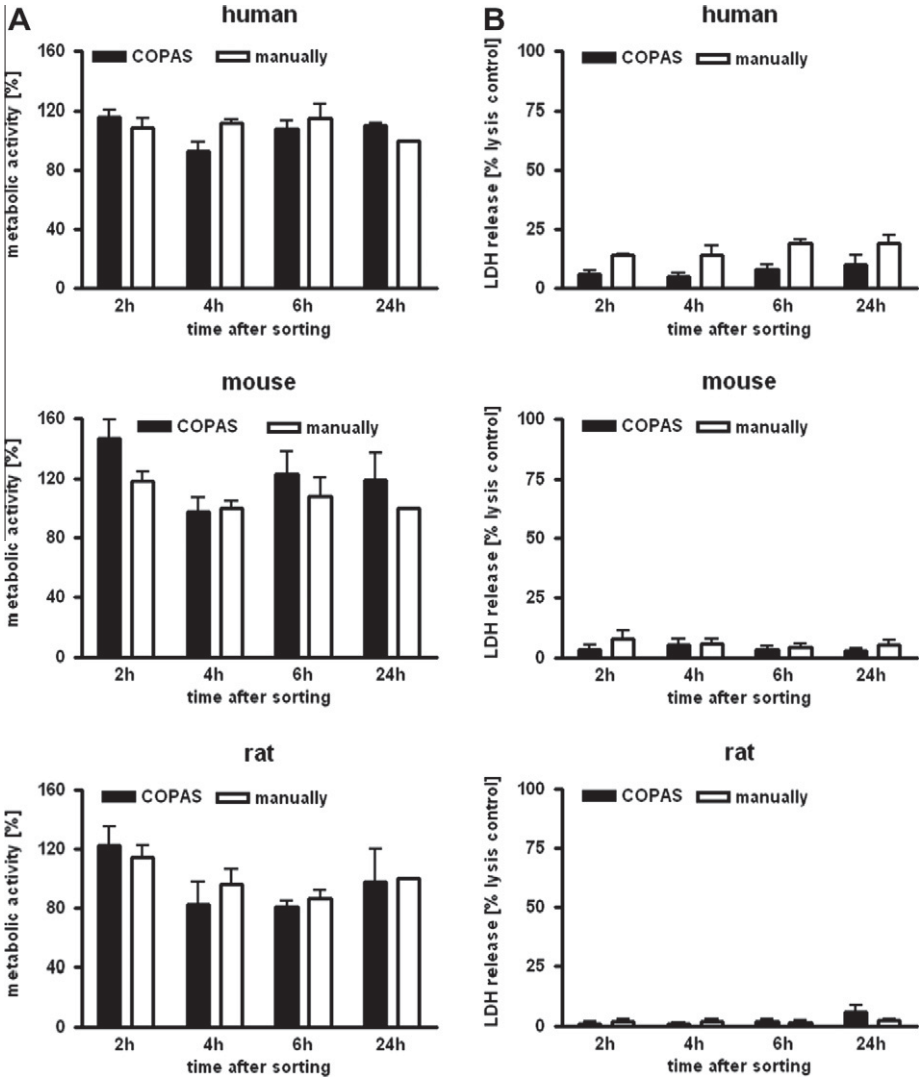

Fig. 2. Viability and cytotoxicity assays. Neurospheres of the same size were sorted by the COPAS instrument (black bars) and manually under a microscope (white bars) in a 96-well plate (one sphere/well in 100 μL proliferation medium) and metabolic activity was measured 2, 4, 6 and 24 h after the sorting with the CellTiter-Blue Assay (Promega) (A) and lactate dehydrogenase activity was determined as a measure for cytotoxicity by CytoTox One Assay (Promega) (B). Shown are the means ± SEM of three to five independent experiments with four spheres per time point in % of the 24 h manually sorted spheres in case of the CellTiter-Blue Assay and in % of the complete cell lysis with triton X-100 for the CytoTox One Assay.

immuncytochemical staining against β(III)tubulin (Sigma–Aldrich, St. Louis, USA). Additionally nuclei were stained with Hoechst 33258 (Sigma–Aldrich, St. Louis, USA). For quantification analyses, we used an ImageJ macro to count the Hoechst 33258 positive nuclei automatically. Neurons were counted manually with the Metamorph analysis software package (Version 7.1.7.0; Universal Imaging Corp., West Chester, PA, USA).

2.6. Dichlorofluorescin (DCF) assay

For determination of intracellular reactive oxygen species (ROS) generation within the sorting process, we employed a flow cytometry method based on the identification of the fluorogenic probe H_2DCF–DA. DCF is a membrane-permeable ROS-sensitive fluorescent probe that does not fluoresce until it is oxidized by intracellular free radicals (Barja, 2002). Cells were washed directly after sorting twice with Hanks' buffered saline solution (HBSS; GIBCO Invitrogen, Paisley, UK) and incubated in HBSS containing 20 μM H_2DCF–DA for 1 h under cell culture conditions. As a positive control neurospheres were incubated with 100 μM tetrachlorohydrochinone (TCHQ) for 1 h because TCHQ induces intracellular redox cycling and thus generates hydroxyl radicals (Zhu et al., 2000). To obtain a single-cell suspension, neurospheres were again washed in HBSS, incubated with accutase at 37 °C for 20 min, and then gently pipetted. The cell suspension was centrifuged (4 °C, 1400 × *g*, 5 min) and the cell pellet was resuspended in 200 μL ice-cold HBSS. Flow cytometry was performed using a FACSCalibur flow cytometer (BD Bioscience) and the FlowJo software analysis package (Tree Star, Inc., Ashland, OR, USA).

2.7. Statistics

All results are means ± SEM of at least three independent experiments. We used the Student's *t*-test for the two-group comparisons (manually sorted vs. COPAS sorted). Significance value was set at $p < 0.05$ (Hays, 1994).

3. Results and discussion

In general, large particles (>200 μm) are sorted with the COPAS instrument depending on their size (measured from the instrument as time of flight = TOF), optical density (EXT) and fluores-

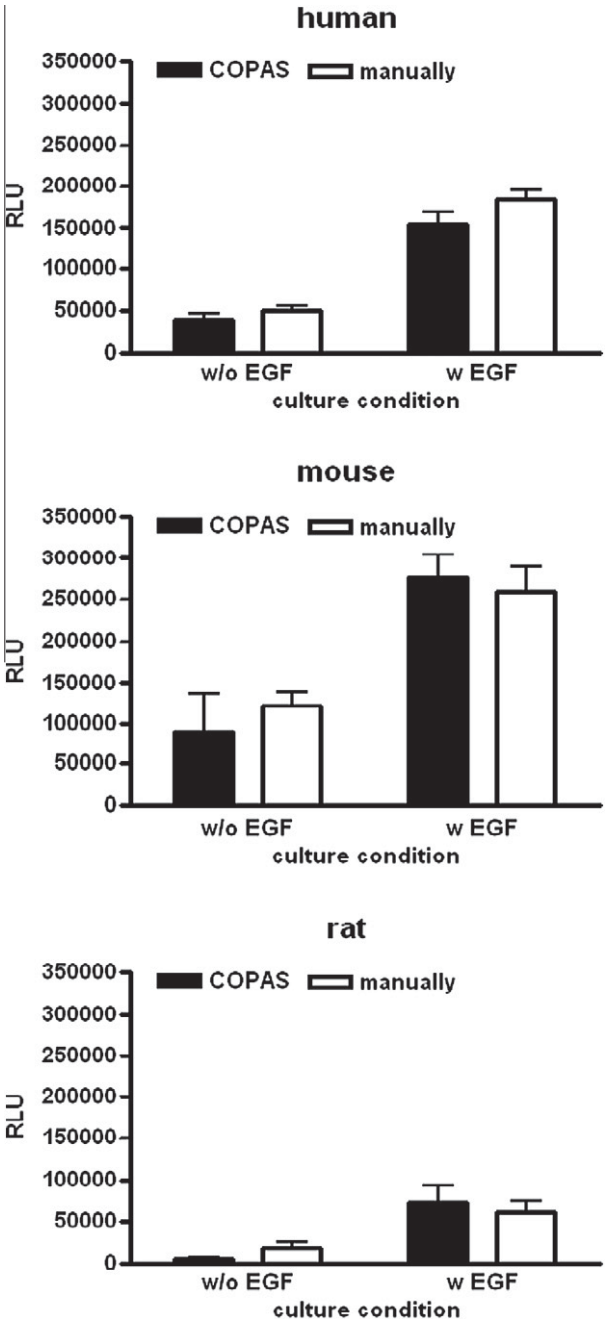

Fig. 3. Proliferation capacity after sorting. Neurospheres of the same size were sorted by the COPAS instrument (black bars) and manually under a microscope (white bars) in a 96 well plate (one sphere/well in 100 µL medium). After 48 h cultivation in proliferation medium with and without 20 ng/mL EGF BrdU was added and its incorporation was measured 16 h later by the luminescence cell proliferation ELISA of Roche. Shown are the means ± SEM of three to four independent experiments with six spheres per exposure in relative luminescence units (RLU).

cence. Sorting and plating of such large items has already been successfully established, e.g. for liver 3D micro tissues, kidney tubules, *C. elegans*, pancreatic islets, *Drosophila* larvae, Zebrafish (embryos and larvae) and tumor histoids earlier (Chen et al., 2010; Ingram et al., 2010; Klapper et al., 2011; Miller et al., 2006; Quinones-Coello et al., 2007; Tjernberg et al., 2008). Here, we adjusted the instrument for neurospheres and investigated if the basic functions of these 3D cell systems, namely viability, proliferation, migration and differentiation, were affected by the sorting process. Moreover, we evaluated if the automated sorting process causes more oxidative stress than cautious single manual pipetting (Matlung et al., 2009; Xie et al., 2007).

For an initial adjustment of settings within the COPAS instrument, we let the COPAS dispense a random sample of cultured neurospheres. By dot plot analyses we created gates for the selected parameters, which exclude small non-spheroid cell clusters, single cells and debris (Supplementary Fig. 1). Next, the COPAS sorted manually-presorted neurospheres (Ø 100–450 µm) into a 96-well plate (one sphere/well). After microscopic diameter evaluation of the individual spheres we correlated each measured diameter with the respective recorded TOF feature in the COPAS PLUS software. We observed a linear relationship between TOF measurements to actual sphere sizes in µm (Supplementary Fig. 2). For all following experiments we used a TOF that equals a sphere diameter between 200 and 400 µm. The selection of this size is crucial because our own data indicates that nutrients, growth factors and/or oxygen penetrate approximately 150–200 µm deep into human neurospheres as within this distance from the sphere surface NPCs reside in their nestin-positive, undifferentiated state (Moors et al., 2009). Moreover, the proliferative capacity decreases if the neurospheres reach a diameter above 1000 µm for human and 700 µm for rodent spheres (unpublished data).

To show that the COPAS instrument measures size with the same accuracy as manual sorting using a binocular microscope, 40–50 human, rat and mouse neurospheres each were sorted and plated with either technique into 96-well plates (one sphere/well). Afterward, diameters of manually vs. COPAS sorted spheres were assessed with the help of the Metamorph software. Human neurospheres sorted manually (M) or by COPAS (C) displayed mean diameters of 330 ± 27 µm and 334 ± 36 µm, respectively. Mouse neurospheres measured mean diameters of 249 ± 20 µm (M) and 247 ± 33 µm (C) and the rat sphere sorting resulted in mean sphere sizes of 231 ± 28 µm (M) and 244 ± 40 µm (C; Fig. 1B).There was no significant difference in mean diameters or the standard deviation between the two sorting techniques (M & C). However, differences in diameters between humans and rodents are obvious. These are determined by the size of the respective starting material in the dishes and can be explained by the observation that average rodent spheres are generally smaller than human spheres (Fig. 1A). Moreover, neurosphere 'chopping' probably plays a role. Two to three days before plating, neurospheres are chopped to receive a larger population of equally sized neurospheres (Fritsche et al., 2011; Svendsen et al., 1998). Mean sphere size in the dishes is thus influenced by the size of neurosphere chopping, time after chopping and speed in proliferation after chopping. The latter can fluctuate, e.g. in dependence on the age of the cultures. However, this does not matter experimentally as long as cell material for samples and controls within one experiment is taken from the same pool of starting material and spheres are within the selected diameter range.

Next, we measured cell viability 2, 4, 6 and 24 h after manually or COPAS assisted sorting and plating of proliferating neurospheres with the Alamar Blue Assay (CellTiter-Blue, Promega), which assesses cellular metabolic activity. From the same samples, cytotoxicity was evaluated by the measurement of LDH leakage. Metabolic activity fluctuated over time between 80% and 140% after sorting with both techniques, but there was no significant difference between manually or COPAS sorted or in-between the three species (Fig. 2A). The same was also true for LDH release into the medium, which was relatively low at all time points (2–10% of complete cell lysis control with triton X-100; Fig. 2B). The highest values were even seen in the manually sorted human spheres indicating that the COPAS sorting might even be gentler to the spheres than manual sorting. However, an approximated difference of 10% in LDH release is probably not biologically relevant, especially with unaffected cell viability data (Fig. 2A).

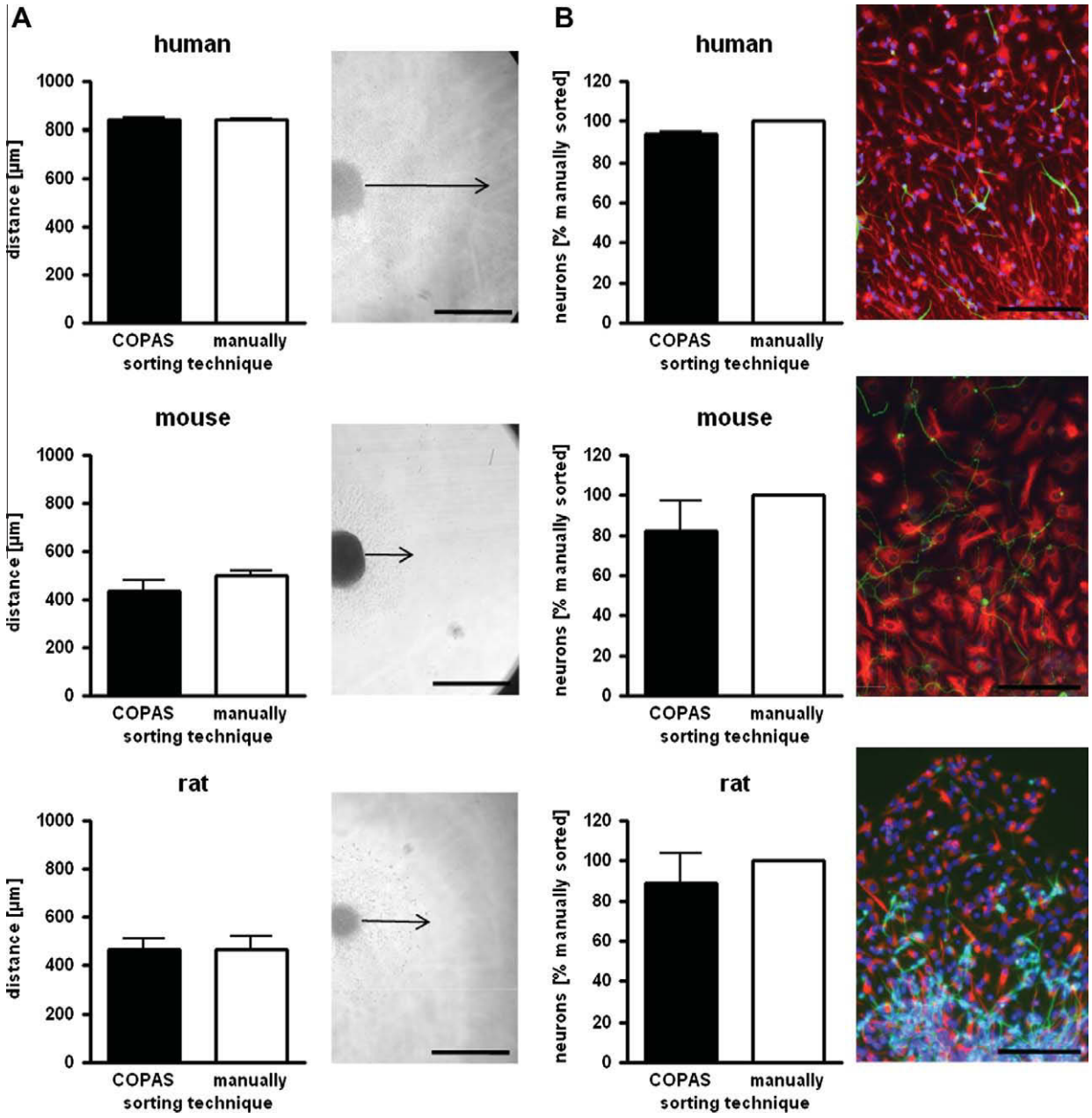

Fig. 4. Migration and differentiation measurement. Neurospheres of the same size were sorted by the COPAS instrument (black bars) and manually under a microscope (white bars) in a poly-D-lysine/laminin coated 8-chamber slide. After 48 h under differentiation conditions the migration distance of the neural progenitor cells out of the sphere was measured (arrow). Scale bar = 500 µm (A). After 72 h of differentiation the proportion of neurons was quantified by immuncytochemistry using a specific antibody against β(III)tubulin (green) and GFAP (red). Cell nuclei were counterstained with Hoechst (blue). Scale bar = 100 µm (B). Shown are the means ± SEM of three to four independent experiments with five spheres per experimental condition and a representive image for each endpoint and species.

In addition to these more general measures of toxicity, we investigated the influence of COPAS sorting on the basic processes of neurosphere development: cell proliferation, migration and differentiation. These endpoints represent the core of 'The Neurosphere Assay', which is employed for evaluation of DNT potential of chemicals (Breier et al., 2010).

Proliferation of neurospheres in presence and absence of EGF was determined by the incorporation of the nucleotide analogue BrdU into DNA of proliferating spheres and subsequent luminescence detection via a peroxidase-coupled antibody in a luminometer. NPCs of the three different species proliferated at different rates. Mouse cells incorporated the greatest BrdU/time (258,209 ± 30,010 RLU (M) and 276,088 ± 28,172 RLU (C)/16 h with EGF), whereas rat NPCs incorporated the least (61,379 ± 14,720 RLU (M) and 74,245 ± 17,637 RLU (C)/16 h with EGF) and human NPCs lay in-between (184,599 ± 9246 RLU (M) and 154,867 ± 13,726 RLU (C)/16 h with EGF). Lack of EGF significantly reduced proliferation in all species, mouse 2.1–3.1-fold, rat 3.3–15.9-fold and human 3.6–4.1-fold indicating that we really measured proliferation (Fig. 3). With regard to the sorting technique, there was no significant difference between M or C sorted and plated neurospheres with or without EGF (Fig. 3).

Upon EGF withdrawal and offer of an extracellular poly-D-lysin/laminin matrix, neurospheres settle down and NPCs migrate radially out of the sphere. Determination of migration distance with the Metamorph program after 48 h provided a very similar migration distance of 466 ± 26 µm for mouse and rat NPCs, whereas human NPCs migrated almost twice this distance (839 ± 12 µm)

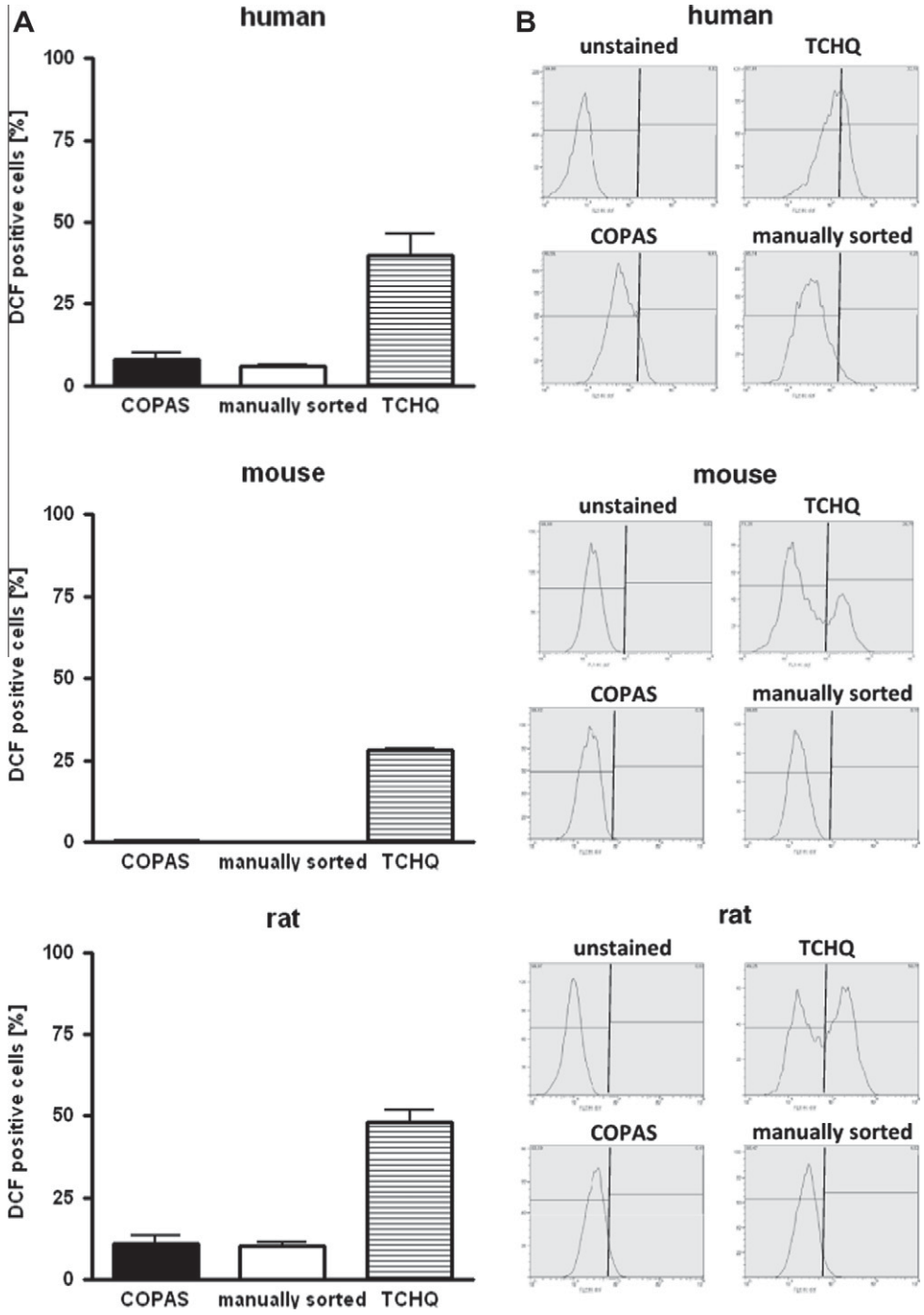

Fig. 5. Assessment of intracellular ROS by DCF assay. Neurospheres of the same size were sorted by the COPAS instrument (black bars) and manually under a microscope (white bars). Manually sorted neurospheres treated with 100 μM TCHQ for 2 h served as positive control striped bars). DCF fluorescence was determined by FACS analysis of singularized cells. Shown are the means ± SEM of four independent experiments (20 spheres per condition and experiment) in % DCF positive cells (A). Representative histograms of the DCF–fluorescence within the NPCs after the different treatments (B).

during the same time. Method of sorting and plating (M vs. C) of neurospheres thereby did not affect migration (Fig. 4A). This most likely means that neurospheres are not stressed during the COPAS plating process because neural migration is sensitively disturbed upon cell stress (Behar and Colton, 2003; Lawton et al., 2010; Sharma et al., 2008).

During migration, cells differentiate into neuronal and glial cells. Therefore, we tested the potential of human, mouse and rat NPCs to differentiate into beta(III)tubulin-positive neuronal cells within 72 h. This time period was chosen as the earliest time-point where a sufficient number of neurons are differentiated according to the experience of the laboratory. Immunocytochemical analyses of the amount of beta(III)tubulin-positive neuronal cells/total nuclei of the migration area indicated no significant differences between M and C sorted spheres (Fig. 4B).

The last parameter we analyzed was the generation of reactive oxygen species (ROS) because shear stress induces ROS in cellular systems (Matlung et al., 2009). As a positive control we included neurospheres treated with TCHQ to induce ROS. FACS analyses of single cell suspensions of M and C sorted neurospheres indicated

that a small amount of ROS generation was found only in human and rat spheres, but that the sorting technique did not influence ROS formation (7.9 ± 2.1% (C) and 5.9 ± 0.2% (M) and 11.3 ± 2.4% (C) and 10.4 ± 1% (M) DCF positive cells, respectively; Fig. 5A). For mouse neurospheres, less than 1% of cells were DCF-positive. Thereby, the gates were set with the help of unstained controls for each species (Fig. 5B). In contrast, TCHQ treatment resulted in 39.9 ± 6.4% of hNPCs, 28.3 ± 0.4% of mNPCs and 48.0 ± 3.8% of rNPCs with positive DCF staining (Fig. 5A). Again, mouse cells exhibited the least ROS generation possibly pointing to species differences in ROS defense capabilities. However, the COPAS sorting induced no additional oxidative stress to sorted and plated neurospheres of all species tested.

In summary, we showed that the COPAS large particle sorter instrument is suitable for sorting and dispensing neurospheres in 96-well plates. None of the parameters tested in COPAS vs. manually sorted and plated neurospheres was affected. It was possible to set up the COPAS to select and dispense same sized spheres as the lab worker by hand. The COPAS sorted spheres were as viable as the manually selected ones and none of the functional endpoints – NPC proliferation, migration and differentiation – which build 'The neurosphere assay' was affected. Moreover, we found no additional ROS generation by COPAS high-throughput sorting. Therefore, our work revealed that the COPAS large particle sorter is, amongst others, a suitable method for high-throughput 3D *in vitro* applications, which contain automated neurosphere sorting and plating.

Conflict of interest statement

None of the authors had any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within 3 years of beginning the submitted work that could inappropriatly influence, or be perceived to influence, their work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2012.04.025.

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Automated neurosphere sorting and plating by the COPAS large particle sorter is a suitable method for high-throughput 3D *in vitro* applications

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Name des Journals:	**Toxicology *in vitro***
Impact Factor:	**3,207**
Anteil an der Arbeit:	**25%**
	Planung, Durchführung und Evaluierung aller Rattenneurosphärenexperimente
Art der Autorenschaft:	**Gleichberechtigte Erstautorenschaft**
Stand der Veröffentlichung:	**zur Publikation angenommen am 24. April 2012**

2.3. Application of the Neurosphere Assay for DNT Hazard Assessment: Challenges and Limitations

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Mechanisms and Predictive Modeling with *In vitro* and *In vivo* Approaches [eingereicht am 09. Januar 2015]

Die Entwicklung des Gehirns ist ein komplexer Prozess, der eine Vielzahl von Entwicklungsprozessen in einer geregelten zeitlichen Abfolge einschließt. Diese Prozesse können in frühe (embryonale) und spätere (fetale) Entwicklungsereignisse eingeteilt werden. Während der embryonalen Organogenese werden die Neuralplatte und das Neuralrohr durch die Differenzierung embryonaler Stammzellen zu Neuroepithel-Vorläuferzellen gebildet. Die Neuroepithel-Vorläuferzellen repräsentieren die neurale Stamm-/Progenitorzellpopulation des sich entwickelnden Gehirns. Wir verwenden neurale Stamm-/Progenitorzellpopulation von Mensch und Nagern (Maus und Ratte), die als Neurosphären kultiviert werden, um fetale Neuroentwicklungsprozesse (Proliferation, Migration und Differenzierung) speziesübergreifend zu untersuchen und adverse Chemikalieneffekte auf diese Prozesse zu evaluieren, und beschreiben, wie diese spezifischen neuroentwicklungsrelevanten Prozesse innerhalb des Neurosphärenassays für die *in vitro* Entwicklungsneurotoxizitätstestung untersucht werden. Dabei heben wir endpunktspezifische Kontrollen für diese Prozesse hervor und verknüpfen diese mit der *in vivo* Situation, um zu demonstrieren, wie *in vitro* Ergebnisse die tatsächliche *in vivo* Situation für Entwicklungsneurotoxizität widerspiegeln können. Zusätzlich beschreiben wir drei Methoden für die Generierung transgener humaner Neurosphären für die biomolekulare Grundlagenforschung. Diese Daten werden kritisch diskutiert, indem die Stärken und Grenzen des Neurosphärenassays für die Ermittlung des Gefahrenpotentials für Entwicklungsneurotoxizität aufgezeigt werden. In der Zukunft wird dieser Assay hinsichtlich seiner biologischen Applikationsdomäne im Hinblick auf die *in vivo* Gehirnentwicklung näher charakterisiert. Solches Wissen wird Gewissheit über die Positionierung des Neurosphärenassays innerhalb einer *in vitro* Testbatterie, die bedeutende Schlüsselereignisse der Gehirnentwicklung abdeckt, geben.

Application of the Neurosphere Assay for DNT Hazard Assessment: Challenges and Limitations

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Running Head:

Neurosphere Assay for DNT Hazard Assessment

Abstract:

Brain development is an integration of developmental processes that take place in a time-dependent manner. These processes can be divided into early (embryonic) and late (fetal) neurodevelopmental events. During embryonic organogenesis, the neural plate and neural tube are formed by embryonic stem cell differentiation into neuroepithelial precursors, which represent the neural stem/progenitor cell population of the developing brain. We use neural stem/progenitor cells of humans and rodents growing as neurospheres to investigate fetal neurodevelopmental events (proliferation, migration and differentiation) across species and to assess adverse effects of chemicals on these processes. We describe how these specific neurodevelopmental processes are analyzed within the “Neurosphere Assay” for developmental neurotoxicity testing *in vitro*. Thereby, we emphasize endpoint-specific controls for these processes and relate those to the *in vivo* situation to demonstrate how *in vitro* outcomes reflect the actual *in vivo* situation for developmental neurotoxicity. In addition, we describe three methods for creating transgenic human neurospheres for basic biomolecular research. These data are critically discussed by pointing out strengths and limitations of the ‘Neurosphere Assay’ for developmental neurotoxicity hazard assessment.

Keywords:

Developmental Neurotoxicity, Neural Progenitor Cell, Brain Development, Species Differences, Human, Mouse, Rat, *In vitro*

1. Introduction:

Brain development is an integration of developmental processes orchestrated in a time-dependent manner [reviewed in 1,2]. These processes can be divided into early (embryonic) and late (fetal) neurodevelopmental events. During embryonic organogenesis, the neural plate and neural tube are formed by embryonic stem cell (ESC) differentiation into neuroepithelial precursors (NEP), which represent the neural stem/progenitor cell (NS/PC) population of the developing brain. During the same period of time, neural crest cells (NCC) also develop, which are precursor cells that give rise to peripheral sensory neurons and glia in addition to other peripheral cell types [reviewed in 3]. In the fetal phase of development, significant organ growth takes place: NEP form radial glia, both types of NS/PC proliferate, radial glia develop scaffolds, cells migrate along those scaffolds and differentiate into young neurons and glia cells [reviewed in 4]. Later maturational stages include elongation of axons and dendrites, specification of neurotransmitters and receptors, formation and pruning of synapses, programmed cell death to eliminate surplus cells, formation of neuronal networks, myelination, and formation of the blood–brain barrier [reviewed in 5 and 6]. These key neurodevelopmental stages are summarized in Figure 1. A multitude of cell types (i.e. ESC, NEP, NS/PC, different neuronal and glial subtypes, endothelial cells) at distinct maturation stages in a brain region-specific manner are involved in these neurodevelopmental processes. These provide numerous targets for compounds acting as developmental neurotoxicants through a variety of different modes of action [MoA; reviewed in 7].

Toxicological testing for regulation of chemicals is currently undergoing a paradigm shift from apical endpoint evaluation in whole animals towards a mechanism-based assessment of compounds' toxicity as determined using multidisciplinary approaches [8]. *In vitro* hazard assessment using cell cultures is one of the main pillars of these approaches. Caution is warranted when choosing appropriate cells for chemical testing, because depending on cell type and culture conditions, results from *in vitro* testing can differ tremendously for the same compounds. Using the examples of *in vitro* testing for developmental neurotoxicity (DNT) endpoints, species [9-11] and cell type [tumor versus primary cell; 12] might influence testing outcome. Moreover, recent advances in tissue engineering have clearly indicated that cells cultured in a conventional 2-dimensional (2D) fashion can differ in their cell physiology from their counterparts growing in 3D [13, reviewed in 14,15]. This observation, which was initially based on data from fibroblasts, also seems to hold true for neural cells [16].

Current international guidelines for DNT testing (OECD Testing Guideline 426, U.S. EPA OPPTS 870.6300) are very resource-intensive when it comes to the number of animals used and the time and costs required [17,18, reviewed in 19 and 20]. In concert with the knowledge on species-specificities of cell and organ responses [21,22], there is international consensus on regarding the need for an alternative strategy for DNT testing with regulatory

acceptance [23,24, reviewed in 25,26]. The “Neurosphere Assay” presented herein is regarded as one tool for such an alternative testing strategy because it mimics specific neurodevelopmental processes (key events) – NS/PC proliferation, migration and differentiation into neural effector cells (neurons, astrocytes and oligodendrocytes) – *in vitro* (Figure 1). In the following sections, we describe how these individual processes are assessed within the “Neurosphere Assay” for *in vitro* DNT testing. In our discussion, we emphasize endpoint-specific controls for these key events and relate those to the *in vivo* situation.

2. The Neurosphere Assay: How to evaluate different processes of brain development *in vitro*

a. Proliferation:

The disturbance of proliferation during brain development leads to substantial alterations of brain morphology including a reduction in the size, weight and volume of the whole brain [27] or individual brain structures [28]. Assessing disturbance of proliferation as an endpoint for DNT is thus of high importance.

We use human second trimester NS/PC growing as 3D cell aggregates called neurospheres (Lonza, Verviers, Belgium). As part of the “Neurosphere Assay” we established a proliferation assay to evaluate the ability of NS/PC to divide and generate new cells by using two different methods for the assessment of proliferation: (1) Bromodeoxy-Uridine (BrdU) incorporation and (2) increase in the diameter of the neurosphere. BrdU-incorporation into the DNA of dividing cells is a direct measure of proliferation. The BrdU Assay is feasible for short exposure times and is a very sensitive method, but results obtained using BrdU incorporation as an endpoint strongly depend on neurosphere size, and inaccurate performance of the assay might lead to false positive results. The alternative method is simpler and assesses proliferation indirectly by measuring the increase in the diameter of floating neurospheres over time. However, this method is less sensitive and only robust changes in proliferation are detected. For this reason, exposure times of at least one week are recommended for this assay [for detailed information on these two methods see 29].

When performing DNT testing in an *in vitro* model it is essential to guarantee that the basic biology of the tested endpoint is functional and the endpoints can be modulated *in vitro* by factors known to modulate them in *vivo*. Therefore, the use of endpoint specific controls for quality assurance is necessary. The selected endpoint specific control for proliferation is growth factor (GF; epidermal growth factor (EGF) and fibroblast growth factor (FGF-2)) withdrawal. NS/PC proliferation is tested both in proliferation medium (B27) including GF and in GF-free medium (B27 w/o EGF and FGF-2). For both methods, GF withdrawal causes at

least 60 to 70% reduction in proliferation relative to controls (Figure 2). NS/PC proliferation is well known to be dependent on EGF and FGF [30-33]. EGF-receptor (EGF-R)-deficient mice develop smaller brains with smaller germinal zones, yet on E17, proliferation in these proliferative areas was not reduced. This might be due to inhibition of proliferation earlier during development and/or compensatory effects of FGF [34]. Yet, EGF-R-deficient animals die within the first 14 days postnatally.

It is essential that an endpoint specific control is specifically acting on the neurodevelopmental process of interest rather than influencing the general viability of the neurospheres. An option for assessing viability is to perform an Alamar Blue Assay, which monitors mitochondrial reductase activity. However, for the endpoint of proliferation, the information obtained by this assay does not accurately reflect cell viability, as sphere diameter and mitochondrial activity are directly correlates because of different cell numbers in different sized spheres [35]. For this reason, e.g. the lactate dehydrogenase (LDH) Assay, which measures cellular membrane integrity, is the preferred method for monitoring cytotoxic effects of compounds in proliferating spheres.

One benefit of the neurosphere model is the opportunity to compare neurodevelopmental processes of different species in parallel *in vitro*, which facilitates extrapolation between species and enables a direct *in vivo*-*in vitro* correlation for rodents. For proliferation, the BrdU and the neurosphere size assay indicated that human, mouse and rat NS/PC proliferate at different rates in culture, and thus present different BrdU incorporation levels after three days (Figure 3A) or different diameter increases after seven days (Figure 3B) of proliferation.

How does NS/PC proliferation respond to DNT compounds *in vitro* and *in vivo*? For the majority of DNT compounds [reviewed in 36 and 37] there is no mechanistic data available for their MoA in humans; therefore, the rodent model is of great value. Comparative human - rodent *in vitro* studies are therefore extremely useful in assessing whether the proposed mechanism of action based on rodent studies is of relevance to humans. Currently, the best-studied DNT compound is methyl mercury (MeHg). Two independent labs demonstrated that MeHg reduced proliferation of rat primary neural cells *in vitro* and in the hippocampi of mice [38] or rats [39] *in vivo*. Furthermore, experimental data from low-dose exposure humans studies demonstrated a decreased mitotic index in lymphocytes, which reflects an inhibition of cell-cycle progression and/or a loss of proliferative capacity [40]. MeHg was also shown to inhibit NS/PC proliferation in the nM range in human umbilical cord blood-derived neural stem cells [41]. Besides MeHg, the antiepileptic drug valproic acid (VPA) is also a well-known developmental toxicant causing autism spectrum disorders in prenatally exposed infants [reviewed in 42]. The mechanisms underlying VPA-induced DNT are not well understood because VPA acts on a multitude of cellular targets [reviewed in 43]. Go and coworkers found that after an injection of VPA in rats at E12, postnatal brain weight was significantly

increased. Moreover, NS/PC isolated from rat brains either exposed prenatally to VPA or treated with VPA *in vitro* displayed increased proliferation *in vitro* [44]. The opposite effects of VPA on proliferation of brain cells *in vivo* were seen when VPA was administered postnatally, and this inhibition of proliferation was recapitulated *in vitro* [45]. These data demonstrate that for some compounds, effects on rodent NS/PC proliferation *in vivo* and *in vitro* correlate well, thus *ex vivo* NS/PC seem to preserve their *in vivo* molecular program. Therefore, it seems likely that primary human NS/PC cultures, such as the above-described primary human neurospheres, will generate data relevant to the human DNT compound hazard on this endpoint. In the context of an alternative DNT testing battery it has to be pointed out that the "Neurosphere Assay" described here is based on fetal (GW 16-20) cells, which will most accurately reflect fetal NS/PC proliferation. This is important to note, because adverse compound effects on NS/PC proliferation might be dependent on the timing of insult [44,45]. How similar hESC- or human induced pluripotent stem cell (hiPSC)-derived NS/PC are to primary hNS/PC and their *in vivo* counterparts and - most importantly - which window of development they reflect, needs to be urgently tested to correctly determine which phase of neurodevelopment they represent in a DNT testing strategy.

b. Migration

Throughout and then after the division of proliferating NS/PC, radial glia as well as postmitotic differentiating cells migrate towards their final destinations in the brain. Several human developmental brain disorders have been associated with disruptions of the migration process, including heterotopia and lissencephaly [46]. Migration defects of specific types of interneurons have also been associated with schizophrenia and epilepsy [47,48, reviewed in 49]. Therefore, an *in vitro* DNT testing battery needs to include a model capable of detecting compound effects on migration (Figure 1).

The "Neurosphere Assay" includes two simple methods to evaluate the ability of NS/PC to migrate simultaneously, mimicking their ability to move from proliferating niches to their final positions in the developing brain. First, migration speed can be assessed by measuring the distance cells migrate over time. A second gauge of successful migration is the evaluation of absolute numbers of cells that migrate out of the neurosphere during this period. To quantify both endpoints, neurospheres are plated onto a poly-D-lysine/laminin coated surface of a well filled with differentiation medium [for more details on the migration protocol see 29]. After adhering to the laminin extracellular matrix, cells spontaneously start to migrate out of the neurosphere [35, Supplementary Material 2]. At the desired evaluation time point (e.g. 24h), phase-contrast pictures of migration areas are taken. Migration distance is evaluated by measuring four radii of the migration area of each neurosphere in perpendicular angles from the edge of the neurosphere to the furthest migrated cells (Figure 4 left). To count the number of migrated cells, neurospheres are fixed with 4% paraformaldehyde and cell nuclei

are stained with Hoechst dye. The total number of Hoechst-positive cell nuclei is then counted in fluorescence pictures of the whole migrating area (Figure 4 right).

These two straightforward ways of evaluating migration allow the comparison of the migration behavior of control cells to compound-exposed cells after a certain exposure time. A close monitoring of the control migration by time-lapse pictures has shown that cells migrating out of the neurosphere exhibit classical migration features required for migration dynamics. Migrating cells present a very active growth cone protrusion moving away from the cell body. This growth cone explores the local microenvironment, and later the cell nucleus is translocated toward the new position (nucleokinesis). In the “Neurosphere Assay” most cells migrate in a radial trajectory using a scaffold built by $GFAP^+$ cells; however, different trajectories and speed rates can also be observed [35, Supplementary Material 2].

Several signaling pathways drive these migration dynamics and one of those is mediated by activation of src kinases [50]. For this reason, the src kinase inhibitor PP2 is used as the endpoint specific control for migration to assess general assay performance (Figure 5A). PP2 does not completely inhibit migration because several other pathways contribute to the migration process, but the distance is decreased to around 60% of control values [50]. It is important to note that because src kinases participate in the regulation of many signaling pathways, one of which is cellular survival [51], a prolonged exposure (72h) to this compound also impacts cell viability (Figure 5B). As there is cross-talk among several pathways involved in migration and the cell cycle [52], compounds affecting signaling pathways involved in migration are also thought to eventually influence cell viability. Therefore, it is important to measure cell viability as early as migration effects are observed when testing the effects of unknown compounds. If migration is reduced as a result of general cytotoxicity, both migration and viability will be reduced, while in the case of specific migration pathway interference, migration will be altered first without showing signs of cytotoxicity. In our experience, this timing effect *in vitro* is true for all studied endpoints, yet migration seems to be very sensitive. In addition to detecting decreases in migration distance, the assay can identify increased migration rates relative to untreated controls [53]. In these cases, general viability pathways are commonly not blocked, and therefore cytotoxic effects are generally not an issue.

Evaluating migration after 24h is also a convenient time point when comparing the effects of compounds on migration of NS/PC of different species, because neurospheres from humans, rats and mice exhibit similar migration dynamics in culture during the first 24h (Figure 6), with a migration distance covering between 400 and 500µm during this period (around 20µm/h). In contrast, during the second 24h (24 – 48h of the migration assay) the migration rates between species differ with 17.2, 9.6 and 1.9µm/h for human, mouse and rat, respectively. Within this time frame, the migration rate of rodent cultures is significantly slower than the

migration speed of the human culture. During the next 24h (48 – 72h of the migration assay) human migration pace further decreases to 7.6µm/h, while rodent speeds stay about the same (Figure 6). It is noteworthy that migration speed during the first 24h in all species is in agreement with real-time observed granule cell migration rates *in vivo* in mice, which range between 14.2 and 26.8µm/h [54]. This *in vitro – in vivo* similarity of migration dynamics strongly underlines the physiological relevance of the neurosphere migration assay.

Most importantly, this migration assay not only accurately reflects the *in vivo* migration process, but also detects migration alterations caused by substances known to disturb human neural migration *in vivo*, like MeHg [54]. Humans developmentally exposed to MeHg suffer from mental retardation and cerebral palsy [55-57]. Their brains are hypoplasic and present many ectopic neurons, indicating disrupted migration among other alterations [reviewed in 58 and 59]. Disturbances of neural migration after developmental exposure to MeHg have also been observed in animal models *in vivo* and *in vitro* [60,61]. In agreement with the literature, the "Neurosphere Assay" detects a significant reduction in NS/PC migration at *in vivo* relevant concentrations of MeHg, which are not cytotoxic [LOAEC= 0.5µM; 35]. In essence, the neurosphere migration assay offers the possibility to study and compare effects of chemicals on NS/PC migration and to elucidate the mechanisms behind migration alterations of compounds in human and rodent cultures.

c. Differentiation:

During the migration period and also afterwards, NS/PC differentiate into neural effector cells to correctly form the different brain regions. NS/PC differentiate into either neurons or glia cells among which the most abundant cell types are astroglial and oligodendroglial cells. The differentiation of NS/PC into these three cell types within the "Neurosphere Assay" will be the subject of the following section. In general, NS/PC differentiation is initiated by growth factor withdrawal and offering a specific extracellular matrix. Therefore, we routinely plate our neurospheres on poly-D-lysine/laminin coated surfaces in medium without growth factors and culture them for 3 (neurons and astrocytes) or 5 days (oligodendrocytes). After this culture period, we fix the neurospheres and perform an immunocytochemical staining for specific epitopes of the different cell types (neurons: ßIII tubulin; astrocytes: GFAP, oligodendrocytes: O4) which are then evaluated by fluorescence microscopy. For mouse NS/PC, the differentiation medium is supplemented with 1% FCS to prevent apoptosis. For a detailed method description, please see Baumann et al. [29].

Neurons:

The differentiation of NS/PC to neurons during brain development is essential for the appropriate cellular composition of the different brain regions and functionality of neuronal networks. Disturbing neuronal differentiation during normal brain development, therefore,

results in structural changes of the brain, which can manifest as behavioral disorders [reviewed in 62]. Thus, the investigation of NS/PC differentiation is another essential endpoint to be evaluated for alternative DNT testing.

We assess the ability of NS/PC to differentiate into neurons within the “Neurosphere Assay” by evaluating the percentage of cells in the migration area that differentiated into neurons. A manual counting of neurons strongly depends on high quality immunocytochemical staining for a clear identification of neurons in the migration area.

To control general assay performance for neuronal differentiation, neurospheres are differentiated in the presence of EGF (20ng/ml) as an endpoint specific control to reliably inhibit NS/PC differentiation to neurons [63]. For a valid endpoint specific control, EGF in the culture medium should reduce the number of neurons to around 20% of the control value (Figure 7). EGF does not reduce viability when administered during differentiation, showing that our endpoint specific control inhibits neuronal differentiation without causing cytotoxicity.

In order to compare neuronal differentiation across species we compared the percentage of neurons in the migration area after 3 days of differentiation under standard differentiation culture conditions in humans, rat and mouse cultures. Quantitative evaluation of the three species revealed statistically significant differences in the amount of differentiated ßIII tubulin+ cells. In human neurospheres approximately 10% of the cells in the migration area were ßIII-tubulin+, whereas in mouse and rat neurospheres, the percentage of ßIII-tubulin+ cells was approximately 5% and 15%, respectively (Figure 8).

When testing chemicals for their DNT potential, how does the endpoint neuronal differentiation measured in *in vitro* reflect *in vivo* data? Kim and coworkers (2009) showed that Bisphenol A (BPA), an endocrine disruptor that potentially disrupts neuronal development [reviewed in 64], promoted neuronal differentiation in the murine immortalized C17.2 progenitor cell line and in primary embryonic hippocampal neurons. These *in vitro* data demonstrating accelerated neurogenesis were reflected by an enhanced dentate gyrus formation in mice on PND1 after administering BPA during pregnancy between E14.5 and 18.5 [65]. Hence, in this study BPA affects murine brain development by altering neuronal differentiation both *in vivo* and *in vitro*. Another study from Go and coworkers [44] demonstrated that VPA induced neuronal differentiation of NS/PC generated from E14 rat cortex after exposure *in vitro* and *in vivo* after a single injection of rat dams with VPA at E12. Studies like these clearly show that an affected neuronal differentiation *in vitro* in primary cells correlates with corresponding *in vivo* data, illustrating that the endpoint of neuronal differentiation is a suitable and important endpoint for *in vitro* DNT testing using NS/PC. Similarly as discussed in the proliferation part of this chapter, we expect each species to be representative of its respective molecular profile, thus human primary cells as described

above resemble human physiology. And again, application of hESC/hiPSC methods for assessing the endpoint neuronal differentiation, especially have to be characterized with regards to developmental time points.

Astrocytes:

Differentiation of NS/PC to astroglial cells during brain development is essential for processes like neuronal migration, neurite guidance and the formation of functioning synapses [reviewed in 66]. Disturbing astroglial cells or neuronal-astroglial interactions during critical periods of brain development (e.g. during neuronal migration) can induce substantial deficits in the function of the central nervous system [reviewed in 67]. Therefore, the assessment of NS/PC differentiation to astrocytes is thought to serve as an important functional endpoint during DNT testing.

We assess the ability of hNS/PC to differentiate into astrocytes by evaluating quantitative and qualitative changes in astrocytes differentiation and maturation using immunocytochemical staining of migrated cells for GFAP. Bone morphogenetic protein (BMP)-7-dependent astroglial differentiation serves as an endpoint specific control [68]. Figure 9 clearly demonstrates that human NS/PC differentiated in the presence of BMP-7 generated more astrocytes with a higher GFAP content and a more mature phenotype with shorter processes and a more stellate-like phenotype.

Comparing the morphology of astrocytes differentiated for 3 days from human, rat and mouse NS/PC reveals similar astroglial morphologies in mouse and rat NS/PC with stellate astrocytes, whereas human NS/PC mainly show elongated astrocytes with more radial glia-like structures (Figure 10). This might indicate that astrocytes differentiated from rodent NS/PC are already more mature after 3 days of differentiation when compared to human NS/PC, which again indicates that human and rodent NS/PC differ in their rate of differentiation and maturation [69,70].

The endpoint of astroglial differentiation for DNT testing has been investigated *in vivo* and *in vitro* earlier. Burry and coworkers observed that postnatal exposure of rats to toluene, one of the known human DNT compounds [reviewed in 37], impaired astrocyte development in the developing brain, which was measured by reduced brain GFAP content in rats treated with toluene daily between PND4 and 10. This toluene-induced GFAP reduction could be the result of reduced astrocyte maturation, diminished astrocyte number or a combination of both. *In vitro* analyses in the same study revealed that proliferation of GD 21 rat cortical astrocytes was reduced after toluene treatment for 24h in a concentration-dependent manner in the absence of cytotoxicity [71]. Similarly, ethanol, which is also a confirmed human developmental neurotoxicant [reviewed in 36], delays the appearance of GFAP during brain development and decreases its expression after prenatal exposure of rats. Radial glial

cultures of rats that were prenatally exposed to ethanol showed a similar reduction in GFAP content [72], and an *in vitro* treatment of rat NS/PC with ethanol resulted in a reduced number of astrocytes after differentiation [73]. Ethanol causes fetal alcohol syndrome (FAS), and there is human evidence that ethanol impairs astrogliogenesis, as evidenced by aberrant neural and glial tissue and other abnormalities in neural and glial migration in post mortem brain of FAS children [reviewed in 74]. These studies demonstrate that results obtained for the endpoint astrogliogenesis both *in vivo* and *in vitro* correlate well, which makes it a valuable endpoint for evaluating the developmentally neurotoxic potential of chemicals.

Oligodendrocytes:

Oligodendrogenesis during brain development is necessary for proper brain function as oligodendrocytes form and maintain myelin sheaths around axons in the central nervous system. Disturbance of oligodendrocyte development may result in demyelination diseases that severely affect neuronal functioning [reviewed in 75].

To study oligodendrogenesis within the “Neurosphere Assay”, the number of O4+ cells/total number of cell nuclei in the migration area is counted and oligodendrocyte morphology is evaluated after five days of differentiation. The endpoint specific control substance chosen to inhibit oligodendrogenesis is BMP-7 (Figure 11), because similar to other BMPs (e.g. BMP-2), BMP-7 induces astrogliogenesis (see above) and inhibits oligodendrogenesis [76,77]. This switch in glial differentiation fate is possibly mediated by SMAD protein signaling [78]. Comparison of oligodendrogenesis across human, mouse and rat neurospheres revealed that after five days of differentiation, hNPC from all species have formed 6-7% O4+ cells/nuclei within the migration area (Figure 12A). However, the maturation stage of oligodendrocytes at this time in culture differs between human and rodent cultures (Figure 12B). Human NS/PC-derived oligodendrocytes are still linear and/or lesser branched with smaller sheath-like structures than their rodent counterparts, which are more branched and form extensive myelin sheaths after five days of *in vitro* differentiation. In addition, the kinetics of oligodendrogenesis differs between species. Murine NS/PC are already differentiated to oligodendrocytes after 2 days, while human NS/PC differentiate to O4+ cells progressively over 5 days of differentiation (Figure 12C). This characterization of oligodendrocyte maturation by morphological appearance (branches/sheath formation) over time demonstrates that murine oligodendrocytes differentiate and mature faster than their human counterparts. These *in vitro* observations reflect what is known *in vivo*: the time span between pre-oligodendrocyte formation and emergence of the first mature oligodendrocytes is 11-12 weeks in humans but only 5 days in rodents [reviewed in 79].

Molecular analyses of myelin basic protein (MBP, for hNS/PC) or myelin oligodendrocyte glycoprotein (MOG, for mNS/PC) mRNA expression support this observation (Figure 12D).

Expression of these maturation markers increases over differentiation time. Human oligodendrocytes mature between day 3 and 5, while murine oligodendrocytes mature between day 1 and 3. The *in vivo* relevance of oligodendrocyte differentiation from NS/PC is displayed by studying the consequence of the thyroid hormone T3 on oligodendrocyte maturation *in vitro*. Thyroid hormone drives oligodendrocyte maturation *in vivo* since hyperthyroid rodents express higher and hypothyroid rodents lower levels of oligodendrocyte maturation genes [80-82]. Similarly, T3 induces MBP and MOG in differentiated human and mouse NS/PC, respectively, with higher potency in human cultures (Figure 12D). This stronger T3-dependent increase of oligodendrocyte maturation markers observed in human compared to murine cultures may be due to a higher T3-responsiveness of human oligodendrocyte maturation genes or an already higher maturation grade of mouse oligodendrocytes at the beginning of the differentiation process with thus limited acceleration of the process.

Besides physiological, hormonal effects on oligodendrocyte maturation (Figure 12D) the literature provides us with the indication that *in vitro* studies concerning oligodendrocyte toxicity have *in vivo* relevance. Eschenroeder and coworkers observed that low doses of the opioid buprenorphine accelerated and increased, but high doses delayed and decreased MBP expression in oligodendrocyte cultures isolated from rat brains [83]. The same dose-dependent effects of buprenorphin on MBP expression were seen *in vivo* in brain lysates of pups treated on embryonic day 7 and sacrificed at PND 12,19 or 26 [84]. A second compound affecting oligodendrocytes during the perinatal period is vanadium. This metal produces hypomyelination during the second postnatal week in rat *in vivo*, a period of the most intense oligodendrocyte development and myelination in this species. This hypomyelination is thought to be caused by oligodendrocyte precursor cell (OPC) apoptosis due to vanadium-induced oxidative stress because vanadium decreases the number of OPC in postnatal brains *in vivo* and causes OPC cell death *in vitro*. In culture, OPC are more sensitive towards vanadium-induced death than astrocytes or mature oligodendrocytes underlining the high susceptibility of OPC towards vanadium that was seen *in vivo* [85]. These two case studies indicate that toxicity measured in primary oligodendrocyte cultures *in vitro* bear a resemblance to the *in vivo* situation. However, isolated oligodendrocyte cultures lack interactions with other cell types like neurons or astrocytes. That this might be of relevance for *in vitro* testing was demonstrated by He and co-workers [86] who induced oligodendrocyte apoptosis with lipopolysaccharides (LPS) only when oligodendrocytes were co-cultured with astrocytes. This example reflects that a representative *in vitro* system for studying DNT with regards to oligodendrocytes needs to integrate interaction of oligodendrocytes with other major brain cell types. Multiple critical pathways for oligodendrocyte survival, proliferation and differentiation are regulated by cell-extrinsic molecules like growth factors, cytokines, hormones and neurotransmitters, which are

expressed by neighboring cells [reviewed in 87]. One system for DNT testing consisting of neurons, astrocytes, oligodendrocytes and microglia are rodent brain aggregate cell cultures [88-90]. Such re-aggregate cultures represent brain cell mixtures of rat brains, thus species-specificities cannot be excluded. Moreover, they are not pure NS/PC cultures, but consist of cells of different maturation states. In contrast, the above-described NS/PC-derived human oligodendrocytes seem to recapitulate oligodendrocyte formation and maturation in co-culture with neurons and astrocytes and thus might be a suitable alternative for oligodendrocyte toxicity testing. However, compounds need to be tested in a system that targets this important cell type.

3. Genetic modification of neurospheres: How to create transgenic cells in 3D *in vitro*

For basic research, transgenic animals have been of great value as they allow study of mammalian gene regulation and their physiological function in a whole organism [91]. They have been utilized to mechanistically investigate a large variety of human diseases [91, reviewed in 92]. Despite the extensive use of these models in basic and pharmaceutical sciences, enormous failure rates in drug development reflect issues of extrapolating data generated in rodents to humans [22,93,94]. Thus, time seems ready for the creation of *in vitro* transgenic human 3D cultures enabling functional studies of individual genes in a human cellular context. For this purpose, different protocols for genetic manipulation of human neurospheres are presented here. Such gene manipulation includes gene knockdown via siRNA or shRNA as well as specific gene overexpression.

Transfection describes a process of introducing foreign nucleic acids into eukaryotic cells [reviewed in 95] with either transient or stable expression of the foreign nucleic acid in the host cell. Transient expression systems are extremely useful for studying elements that regulate immediate gene expression or for retrieving experimental results within a short time frame. The expression of the externally introduced nucleic acid may be detected for up to 4 days depending on the specific characteristics of the introduced nucleic acid, the cell type and doubling time of cells [reviewed in 95]. The most prevalent transfection method is lipofection. Lipofection has been proven to efficiently deliver molecules from small oligonucleotides to entire proteins into the cell [96]. Although lipofection is most commonly used and also is effective for transfection of human neurospheres (data not shown), we use an alternative transfection method called nucleofection for transfection of neurospheres. This method uses chemical and physical procedures to transfer exogenous material into the cell and usually results in a higher transfection efficiency than common lipofection reagents [97] and higher cell viability after transfection compared to electroporation [98]. We performed transfections with the 4D-Nucleofector® X unit (Amaxa, Lonza Cologne, GmbH) and the Nucleofector® X solutions P3 for primary cells. The correct electric pulse for the NS/PC culture was established by transfecting with the pmaxGFP® vector (Figure 13; Amaxa, Lonza

Cologne, GmbH; for detailed information of this method see 4D-Nucleofector™ instructions, Lonza), which produced observable green fluorescence several hours after transfection.

Viral vectors were originally developed as an alternative to transfection of naked DNA for molecular genetics experiments. Compared to traditional transfection, transduction can ensure that nearly 100% of cells are infected without severely affecting cell viability [99]. Furthermore, some viruses, e.g. lentiviruses, integrate into the cell genome ensuring stable gene expression [99]. For safety reasons, lentiviral vectors never carry the genes required for their replication. To produce a lentivirus, three plasmids are transfected by calcium phosphate precipitation into a so-called packaging cell line, HEK 293FT. Two plasmids, the gag-pol and VSV-G expression vectors, which are generally referred to as packaging plasmids, encode the virus proteins. A third plasmid contains the gene of interest [100]. For NS/PC infection with viral particles, neurospheres are chopped with the McIllwain tissue chopper [29] into 0.1mm pieces. These pieces are incubated with 10,000 lentiviral particles/cell in DMEM/F12 medium containing 100ng/ml EGF, 50ng/ml FGF and 0.02mg polybren. To select for transduced cells, the selection antibiotic puromycin (0.25µg/ml) is added to the medium starting 3 days after infection. Control of the efficiency of genetic manipulation is subsequently achieved by PCR, western blotting and/or immunocytochemistry. Because of the stable insertion of nucleic acids, cells can be propagated and cultures used for experiments for several weeks (Figure 14).

In contrast to lentiviruses, adenoviral DNA does not integrate into the genome and is not replicated during cell division [reviewed in 101]. Nevertheless, due to their high transduction efficiency, broad host range ability to infect non-dividing cells, and potential for generating high titer virus, recombinant adenoviral vectors are one of the most efficient viral vectors for gene delivery *in vivo* and *in vitro* [reviewed in 101]. For more detailed information on adenovirus production, the reader is referred to Yuan et al. [102] and Weggen et al. [103]. Again, as it is done for lentiviral transduction, human neurospheres are chopped to 0.1mm pieces and infected with different virus concentrations (50 – 1000 virus particles/cell). This titration of virus particles is necessary to find the best ratio of virus particles to cells. Four hours after transduction, the medium is completely changed to normal proliferation medium (Figure 15). Two days after infection, spheres are ready to use for experiments and nucleic acid delivery can be monitored by western blotting and/or immunocytochemistry. Because adenoviral transduction does not lead to insertion of the foreign nucleic acid into the host genome, products get lost during cell division. This is the reason why experiments with adenoviral transduced cells can only be performed during the course of a few days. Specific times for experiments have to be tested for each nucleic acid because cellular half-life depends on the individual product.

The choice of method for gene manipulation of neurospheres depends on the experimental question asked and the transfection/transduction efficacy of the respective nucleic acid. This has to be tested individually. However, for short-term experiments, nucleofection is the fastest method for generating transient transgene neurospheres, and thus, the desired method in cases where the transfection efficacy is sufficiently high (Table 1).

4. Conclusions and future directions:

Compared to tumor cells grown in 2D, 3D neurospheres are a fairly sophisticated cell system. They consist of primary cells, comprise or produce the four major neuroectodermal cell types of the human brain (NS/PC, neurons, astrocytes, oligodendrocytes), are self-organized, and can be cultured in two differentiation stages (proliferating and differentiating). Moreover, they can be generated from different species including humans. Species-overarching investigations of molecular and cell biological responses are of substantial importance for the translational aspect of basic science as well as for efficacy and toxicity testing in pharmacology and toxicology. Molecular species-specificities seem to be one reason for the failure of novel drug development for diseases like sepsis and ischemic stroke [93,94]. Therefore, the data presented in this chapter focused on basic species-dependent properties of neurodevelopmental processes analyzed with the “Neurosphere Assay” *in vitro*. Moreover, relevance to the *in vivo* situation of *in vitro* data generated with NS/PC was presented.

With regards to the developing brain, primary human cultures are currently the closest model to the human *in vivo* situation. This is supported by the observations that rodent brain cells taken out of the *in vivo* situation placed into a tissue culture dish maintain their signaling functions or their responses towards xenobiotics [e.g. 39,45,44,104,105], and thus, primary human cells are thought to behave similarly when removed from the *in vivo* to the *in vitro* situation. Although NS/PC generated from human embryonic (hESC) or human induced pluripotent stem cells (hiPSC) are also promising methods for human cell-based DNT testing, the *in vivo*-relevance of these models for neurodevelopment, especially with regards to developmental timing and cell type differentiation, i.e. formation of oligodendrocytes, remains an outstanding question.

Nevertheless, despite the advantages of such 3D *in vitro* systems, one still has to be aware that these are models - as are animals and other cell-based assays. Every model has its strengths and limitations. The limitations of *in vitro* models in conventional culture are mainly their lack of physiological cell and organ context as this creates artificial pharmaco-/toxicokinetics. Thus, the whole issue of absorption/distribution/metabolism/excretion (ADME) is not considered in *in vitro* testing situations. This can be circumvented when human *in vivo* target organ concentrations of compounds as well as their *in vitro* kinetics are known then

providing the opportunity to model realistic exposure scenarios *in vitro*. Another approach to implement kinetics *in vitro* is the 'Organ-on-a-Chip' technology [106, reviewed in 107,108]. The use of human cells in this approach enables better realization of human ADME. Another limitation of the neurosphere system is the lack of some cell types. Neurospheres do not contain microglia or endothelial cells. The latter is an especially crucial factor because in conjunction with astrocytes, endothelial cells form the blood-brain-barrier. This important barrier determines internal brain exposure towards compounds as well as towards endogenous mediators like hormones or cytokines. Although in these self-organized co-culture models one does have interactions between different cell types – neurons, astrocytes and oligodendrocytes – the deficiency in microglia and endothelial cells has to be taken into account. In addition, there is evidence that identical brain cell types differ in their function from one brain region to another [e.g. reviewed in 109]. These brain region-specific differences can easily be studied by cell preparations from different brain areas in rodents. Such studies have identified region-dependent susceptibility of developmental astrocyte proliferation towards MeHgCl: while this compound inhibits hippocampal astrocyte proliferation, cortical, cerebellar and brain stem astrocytes proliferation is not specifically inhibited by MeHgCl [110]. Modeling human brain region-specific cell characteristics *in vitro* is not possible based on current knowledge. In the future, with more information on the physiological factors responsible for brain region-specific cell development and function, it might be possible to also model this aspect in a dish by manipulating hESC or hiPSC accordingly. Last, these cultures cannot think, feel and associate, so they can only mimic neurodevelopmental processes. However, with additional, complementary assays, filling the biological application domains not covered by the "Neurosphere Assay", i.e. neuronal network formation and electrical network activity measurements, a battery of tests can be created enveloping the major key events of human brain development in a dish and thus – although still not thinking – generate an *in vitro* scenario that is able to give a certain mechanism-based level of certainty for DNT assessment. Ideally, such a battery would be embedded into the theoretical frame of the 'Adverse Outcome Pathway' (AOP) concept [111], which currently is receiving significant attention from regulatory agencies worldwide. AOPs for DNT are still sparse and thus urgently needed. The cross-species evaluation of signaling pathways, which is possible with the neurospheres presented in this chapter, is highly suited to fill the data gap on translation from rodents to humans, an important aspect within the AOP concept.

Also for basic biomedical research these cultures can be of great value, especially with the option of creating transgenic neurospheres as described above. This technique is (i) much faster and more cost-effective than first creating a transgenic animal and subsequently preparing neurospheres from the pups; and (ii) is based on the species typically of greatest regulatory concern – humans. The obvious limitation of this approach is, however, that *in*

vitro gene silencing never produces a complete knockout as in an animal model, but is restricted to a knock-down with various degrees of gene repression.

This chapter discusses the strengths and limitations of the “Neurosphere Assay” for DNT hazard assessment. In the future, this assay will be further characterized for its biological application domain with regards to human *in vivo* brain development. Such knowledge will provide confidence for its position in a DNT *in vitro* testing battery that covers major key events in neurodevelopment for regulatory implementation.

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Table Captions:

Table 1: Comparison of different transgenic modification methods in NS/PC in terms of experimental applicability

Tables:

Table 1:

	Lentivirus	Adenovirus	Nucleofection
Virus production time	**2 weeks**	**1 week**	**---**
Timespan until experimental use after transduction/transfection	**1 week**	**2 days**	**1 day**
Timespan to perform experiments	**Several weeks**	**A few days**	**A few days**

Figure Captions

Figure 1: Basic processes of brain development necessary for proper organ function. Neural Progenitor Cells (green) proliferate to provide an excess amount of cells, which then migrate and differentiate into neurons (purple) and glia (yellow). These form synapses (red) and excess cells undergo apoptosis (grey). When these processes happen in the appropriate and coordinated way, functional neuronal networks form (olive). With courtesy from William Mundy, U.S. Environmental Protection Agency and John Havel, SRA International, Inc..

Figure 2. Quality control of the endpoint proliferation in human NS/PC. Proliferation was assessed by performing a BrdU Cell Proliferation ELISA (Roche, black bars) or measuring the diameter increase (grey bars). Therefore, 6 neurospheres were plated in a 96-Well plate (1 sphere per well) in normal proliferation medium with (control) or without growth factors (w/o GF) for 72h. For assessment of BrdU incorporation, 16h prior to the end of the experiment BrdU was added to the spheres. The assay was performed according to the manufacturer's instructions. For the assessment of the diameter increase, the diameter was measured on day 0 and day 3. Data (% of control) is shown as mean of 3-4 independent experiments ± SEM. * p-value ≤ 0.05 (students t-test) was considered as significant compared to control.

Figure 3. Species comparison of the endpoint proliferation. A) Proliferation was assessed by performing a BrdU Cell Proliferation ELISA (Roche). Control data (RLU) of 3-5 independent experiments is shown as mean ± SEM. * p-value ≤ 0.05 (students t-test) was considered as significant between the species. **B)** Phase-contrast images of human, mouse and rat neurospheres taken after 0 days (0 d) or 7 days (7 d) of proliferation, scale bar = 100µm.

Figure 4. Measuring migration within the "Neurosphere Assay". Left) Phase-contrast image of the whole migration area of a human neurosphere after 24h of culture on a PDL/laminin coated surface of a well filled with differentiation medium. Arrows exemplify the assessment of the migration distance by measuring four perpendicular radii from the neurosphere edge to the end of the migration area. **Right)** Fluorescent picture of the whole migration area of the same human neurosphere, where migrated nuclei stained with Hoechst can be counted, scale bar = 100µm.

Figure 5. Quality control of the endpoint migration in human NS/PC. Human neurospheres were cultured for 24 or 72h on a PDL/laminin coated surface of a well filled with differentiation medium (control) or differentiation medium with 10µM PP2. Migration distance was measured in phase-contrast images of 5 neurospheres per assay. Viability of the same spheres was measured by Alamar Blue Assay. Bars represent the mean of 4

independent experiments ± SEM in % of control. * p-value ≤ 0.05 (students t-test) was considered as significant versus control.

Figure 6. Species comparison on NS/PC migration distance over time. Human, mouse and rat neurospheres were cultured for 72h in differentiation medium on PDL/laminin coated wells. Phase-contrast images were taken every 24h and migration distance was measured in 5 neurospheres per assay. Bars represent the mean of 4 independent experiments ± SEM. Statistical analysis was performed by multiple t-test with Holm-Sidak multiple comparison with the GraphPrism 6 program, and significant threshold was established at $p<0.05$. * was considered statistically significant different versus human value of the same time-point. # [54]

Figure 7. Quality control of the endpoint neuronal differentiation in human NS/PC. A) Neuronal differentiation was assessed by immunocytochemical staining of migrated human NS/PC after 3 days of differentiation for the neuronal marker ßIII tubulin and Hoechst for nuclear counterstaining. Therefore, 5 neurospheres were plated in one well of a 8-chamber slide in normal differentiation medium (control) or differentiation medium with 20ng/mL EGF for 72h. **B)** For the assessment of NS/PC viability an Alamar Blue Assay was performed according to the manufacturer's instructions after 3 days of differentiation. Data (% of control) is shown as mean of 4 independent experiments ± SEM. * p-value ≤ 0.05 (students t-test) was considered as significant compared to control.

Figure 8. Species comparison of the endpoint neuronal differentiation. A) Neuronal differentiation was assessed by determining the percentage of neurons in the migration area after immunocytochemical staining for ßIII tubulin and cell nuclei. Control data (% Neurons of total nuclei) of 4 independent experiments is shown as mean ± SEM. * p-value ≤ 0.05 (students t-test) was considered as significantly different between the species. **B)** Fluorescent images of human, mouse and rat neurospheres taken after 3 days of differentiation (blue: cell nuclei; red: ßIII tubulin positive cells), scale bar = 100µm.

Figure 9. Quality control of the endpoint astrocyte differentiation in human NS/PC. Astrocyte differentiation was assessed by immunocytochemical staining of migrated human NS/PC after 7 days of differentiation for the astrocyte marker GFAP and Hoechst for nuclear counterstaining. Therefore, 5 neurospheres were plated in one well of a 8-chamber slide coated with PDL/laminin in normal differentiation medium **(A)** or differentiation medium with 100ng/mL BMP-7 **(B)** for 7 days (blue: cell nuclei; red: GFAP positive cells), scale bar = 100µm.

Figure 10. Species comparison of the endpoint astrocyte differentiation. Astrocyte differentiation was evaluated by immunocytochemical staining of the migration area after 3 days of differentiation for the astrocyte marker GFAP (red) and cell nuclei (blue), scale bar = 20µm.

Figure 11: Quality control of the endpoint oligodendrogenesis in human NS/PC. Human neural progenitor cells were plated onto PDL/laminin coated 8-chamber glass slides in normal differentiation medium (control) and differentiation medium with 100ng/mL BMP-7 for 5 days. Mitochondrial activity was measured by Alamar Blue Assay. Slides were fixed and immunocytochemically stained for O4. Data is shown as mean of 5 independent experiments ± SEM. * p-value ≤ 0.05 (student t-test) was considered as significant.

Figure 12. A+B) NS/PC were plated onto poly-D-lysine/laminin coated 8-chamber glass slides in differentiation medium and were differentiated for 5 days. Afterwards they were fixed and immunocytochemically stained for O4. **A)** Percentage of oligodendrocytes in human, mouse and rat neurospheres was evaluated in the migrating area after 5d of differentiation. (n=5 each; mean ± SEM; no statistical differences by t-test; p≤0.05). **B)** Stages of maturation in human, murine and rat NS/PC after 5 days of differentiation (green: O4, blue: Hoechst), scale bar = 50µm. **C**) Neural progenitor cells were fixed at day 1, 2, 3, 4 and 5 of differentiation, stained for O4 and the percentage of oligodendrocytes in the migration area was calculated (n=4 each; mean ± SEM). **D**) Per condition 3x 10 NS/PC were plated onto PDL/laminin coated 24 well plates in presence or absence of 3nM T3. RNA was collected and isolated at day 1, 3 and 5 of differentiation (RNeasy Kit, Qiagen) and quantitative real time RT-PCR was performed. Data is shown as mean ± SEM (n=3, student t-test, *p≤0.05, DOD: days of differentiation.

Figure 13. Transfection of human NS/PC with the 4D-Nucleofector® X unit. The flow diagram shows the various work steps for transfecting human NS/PC by nucleofection. Furthermore the transfection of human NS/PC with green fluorescent protein (GFP) is shown. Expression of GFP is demonstrated by fluorescent microscopy 24h after transfection, scale bar = 100µm.

Figure 14. Lentiviral transduction of human NS/PC. The flow diagram shows the various work steps for lentiviral transduction. Furthermore the lentiviral transduction of human NS/PC with green fluorescent protein (GFP) is shown. Expression of GFP is demonstrated by fluorescent microscopy 48h after transduction, scale bar = 100µm.

Figure 15. Adenoviral transduction of human NS/PC. The flow diagram shows the various work steps and adenoviral transduction of human NS/PC with Amyloid Precursor Protein (APP). 48h after transduction with different virus particle concentrations, human NS/PC lysates (20µg protein/lane) were analyzed for APP expression by Western blot using an APP-specific antibody. A GAPDH-specific antibody was used as a loading control.

Figure 1:

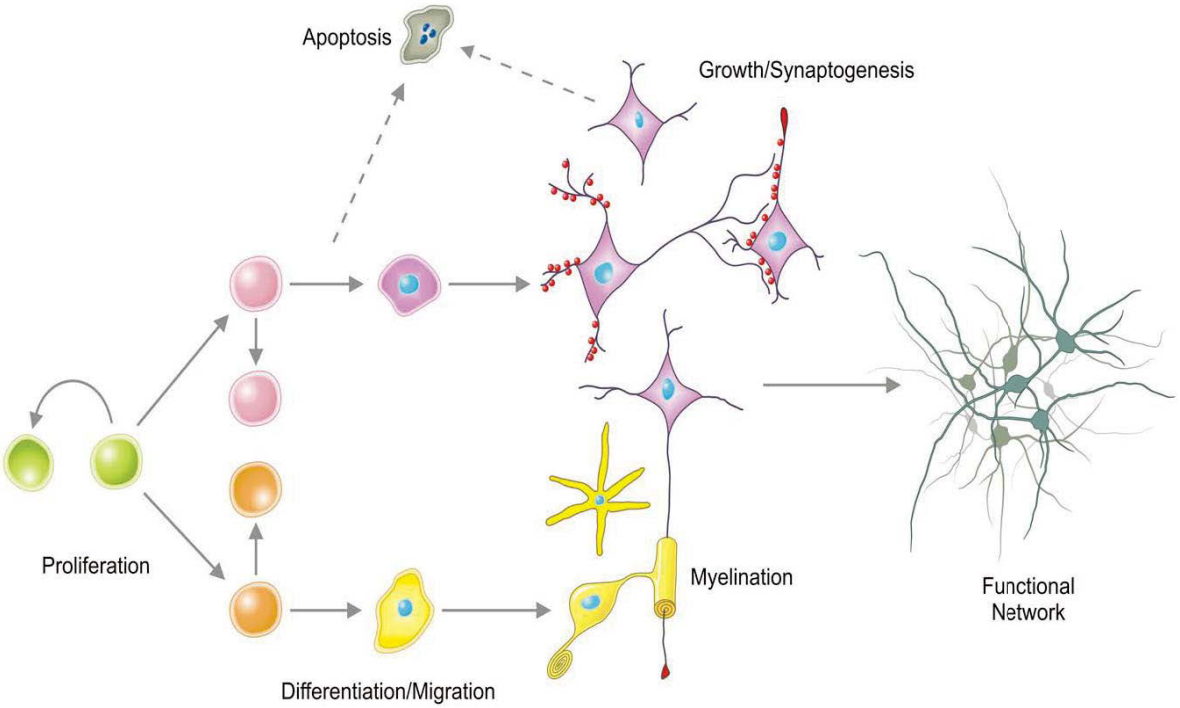

Apoptosis
Growth/Synaptogenesis
Proliferation
Myelination
Functional
Network
Differentiation/Migration

Figure 2:

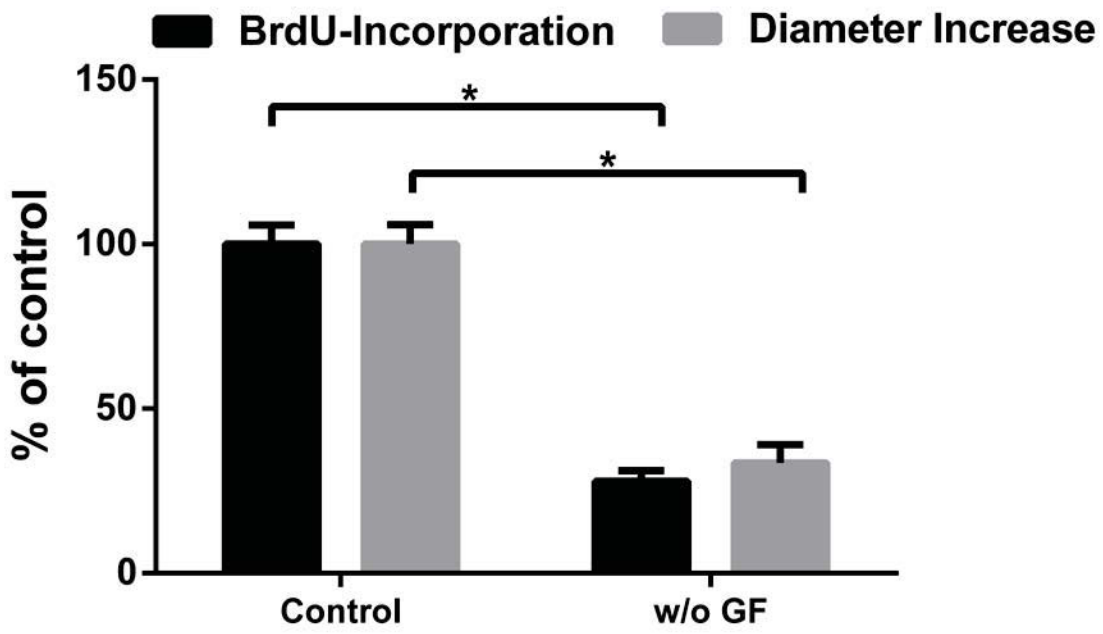

BrdU-Incorporation
Diameter Increase
150
100
50
0
% of control
*
*
Control
w/o GF

Figure 3:

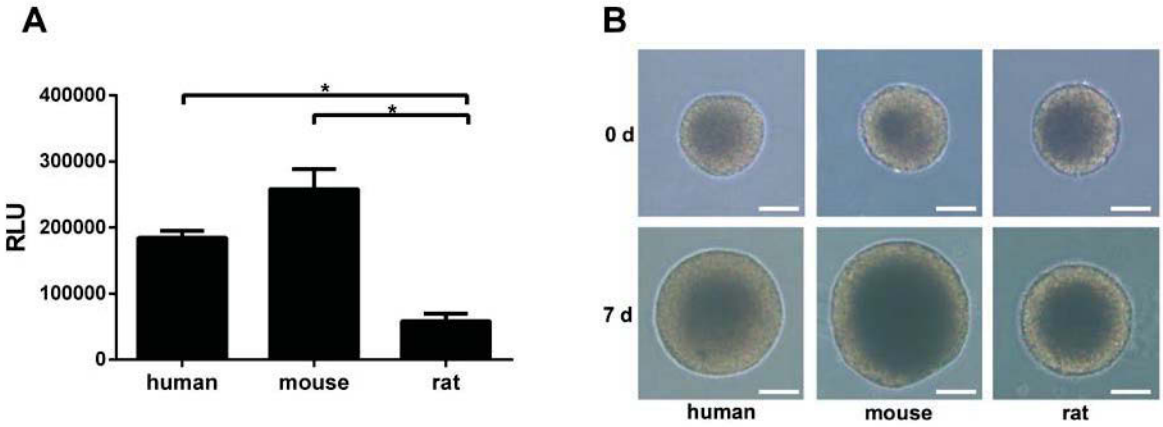
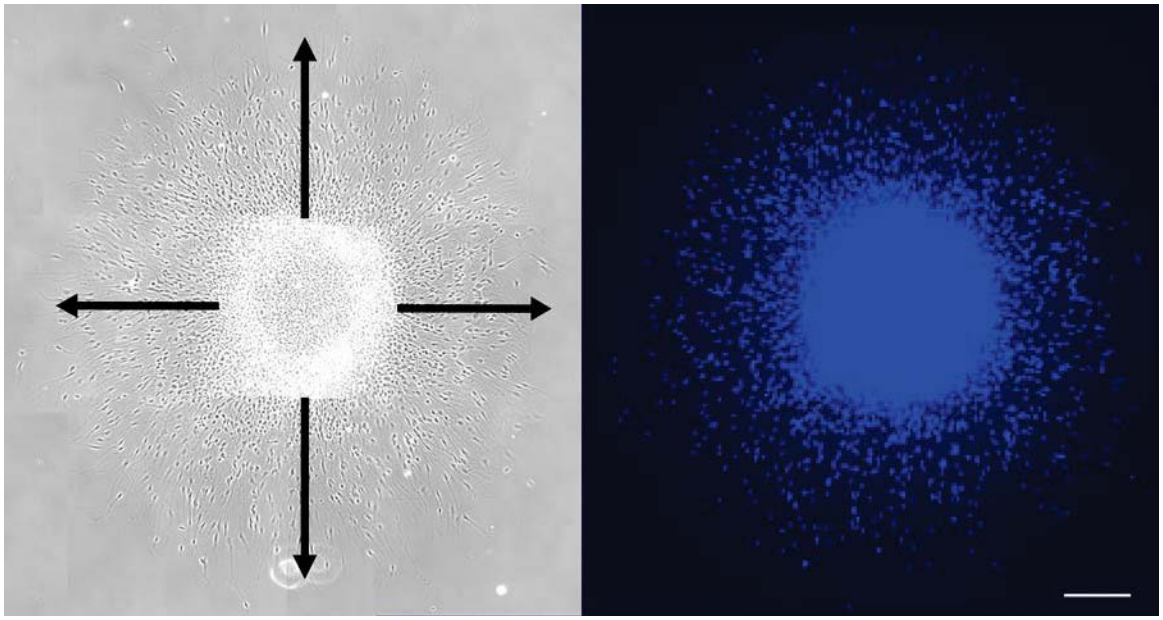

A
400000
300000
200000
100000
0
RLU
*
*
human
mouse
rat
B
0 d
7 d
human
mouse
rat

Figure 4:



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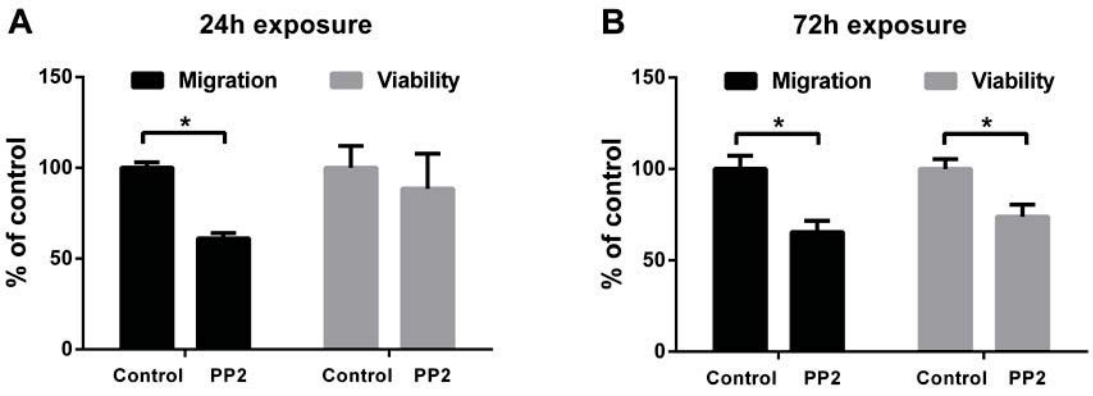
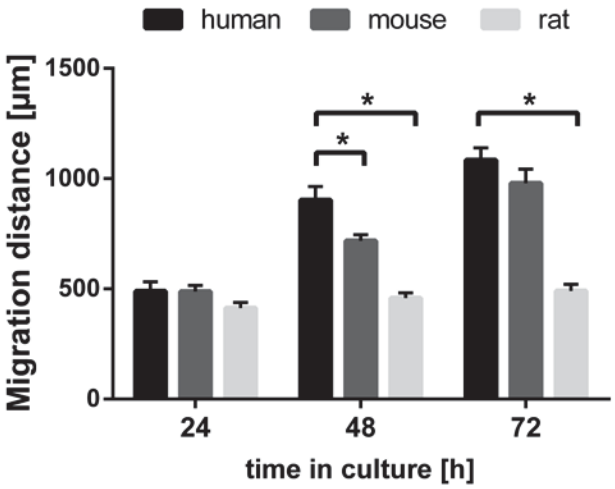

A
24h exposure
Migration
Viability
% of control
150
100
50
0
*
Control
PP2
Control
PP2
B
72h exposure
Migration
Viability
% of control
150
100
50
0
*
*
Control
PP2
Control
PP2

Figure 6:



species	conditions and migration rate [µm/h]			
	in vivo			
mouse	14.2 - 26.8 µm/h [#]			
	in vitro			
	0-24h	**24-48h**	**48-72h**	
human	20.4	17.2	7.6	µm/h
mouse	20.3	9.6	10.9	µm/h
rat	17.2	1.9	1.3	µm/h

Figure 7:

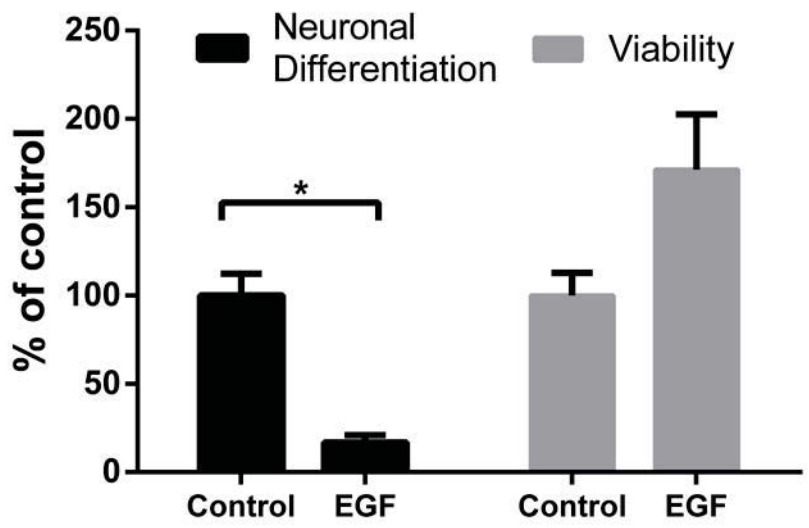

250
200
150
100
50
0
% of control
Neuronal Differentiation
Viability
*
Control
EGF
Control
EGF

Figure 8:

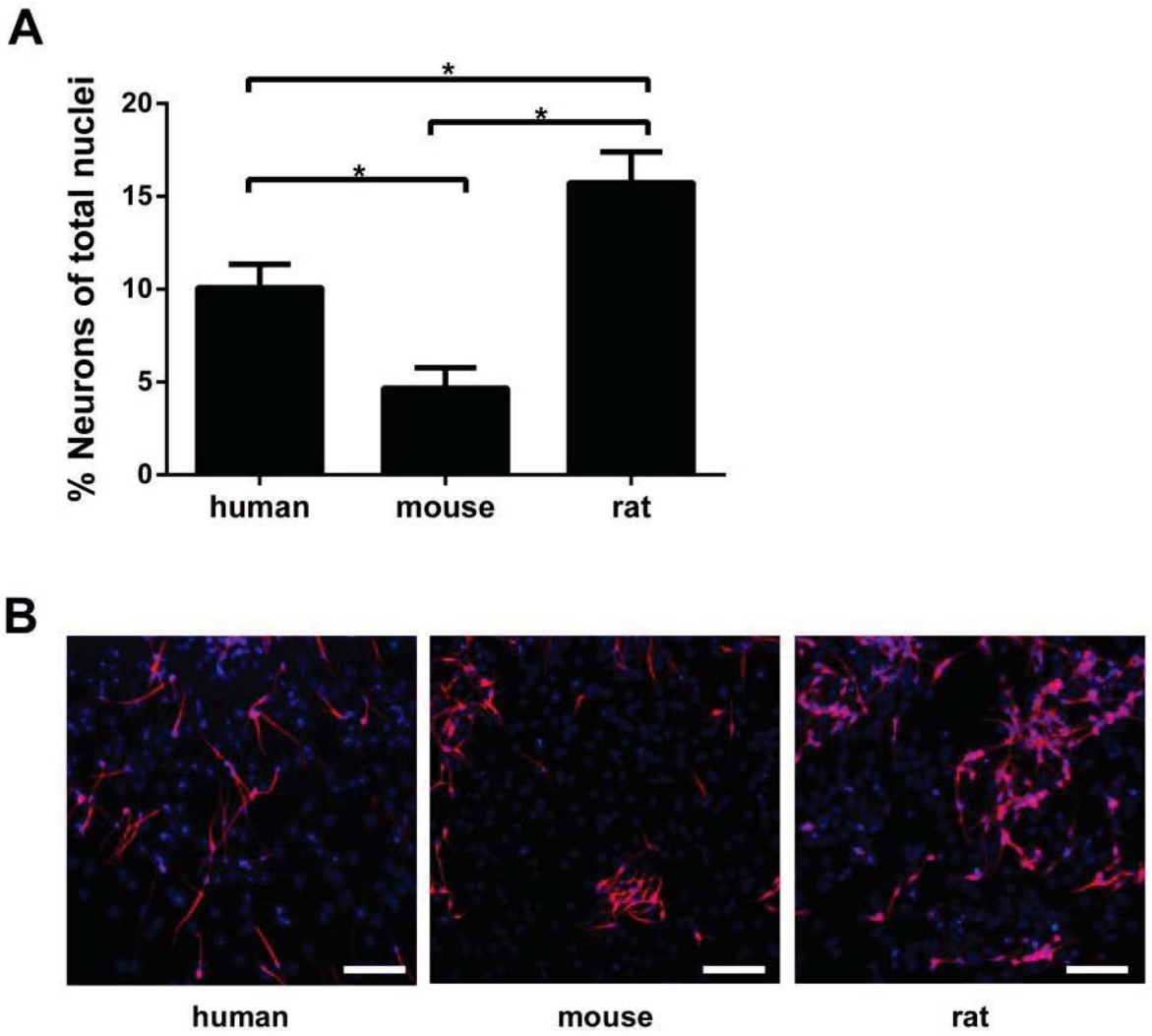
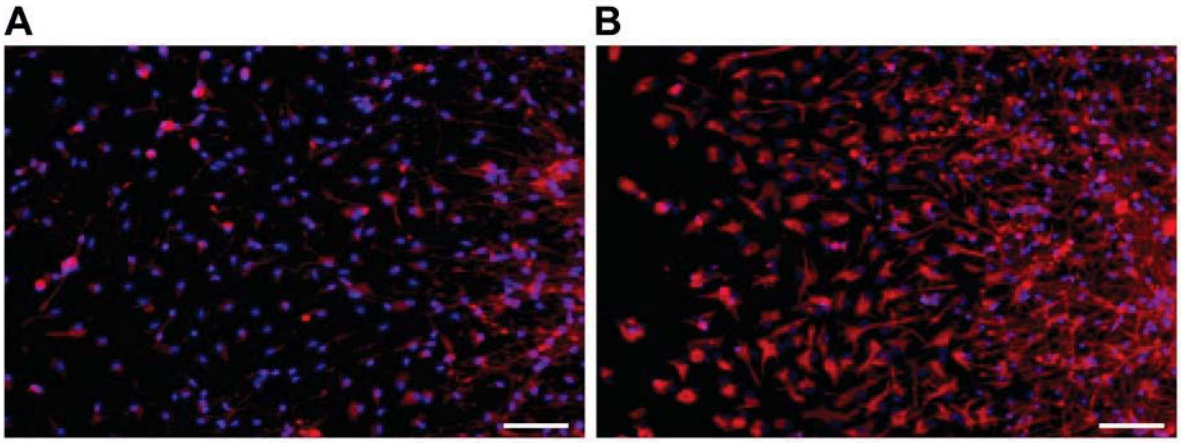

A
% Neurons of total nuclei
20
15
10
5
0
*
*
*
human
mouse
rat
B
human
mouse
rat

Figure 9:



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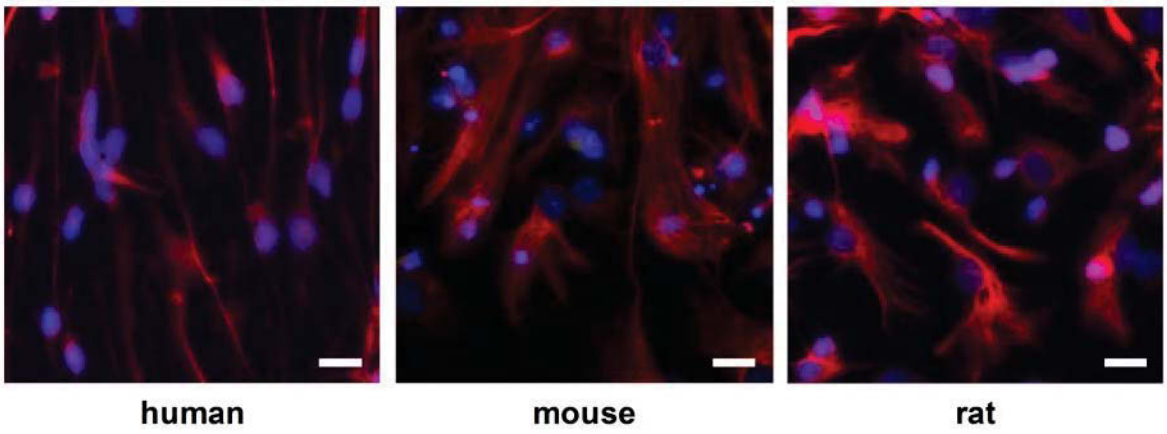

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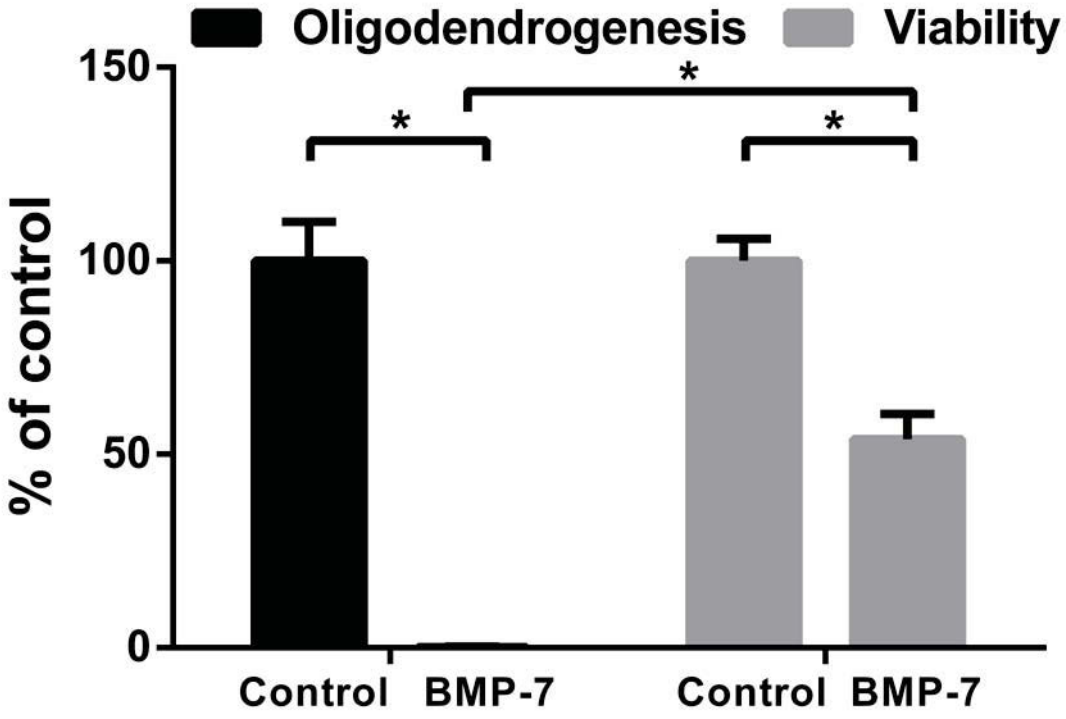

Oligodendrogenesis
Viability
150
100
50
0
% of control
*
*
*
Control
BMP-7
Control
BMP-7

Figure 12:

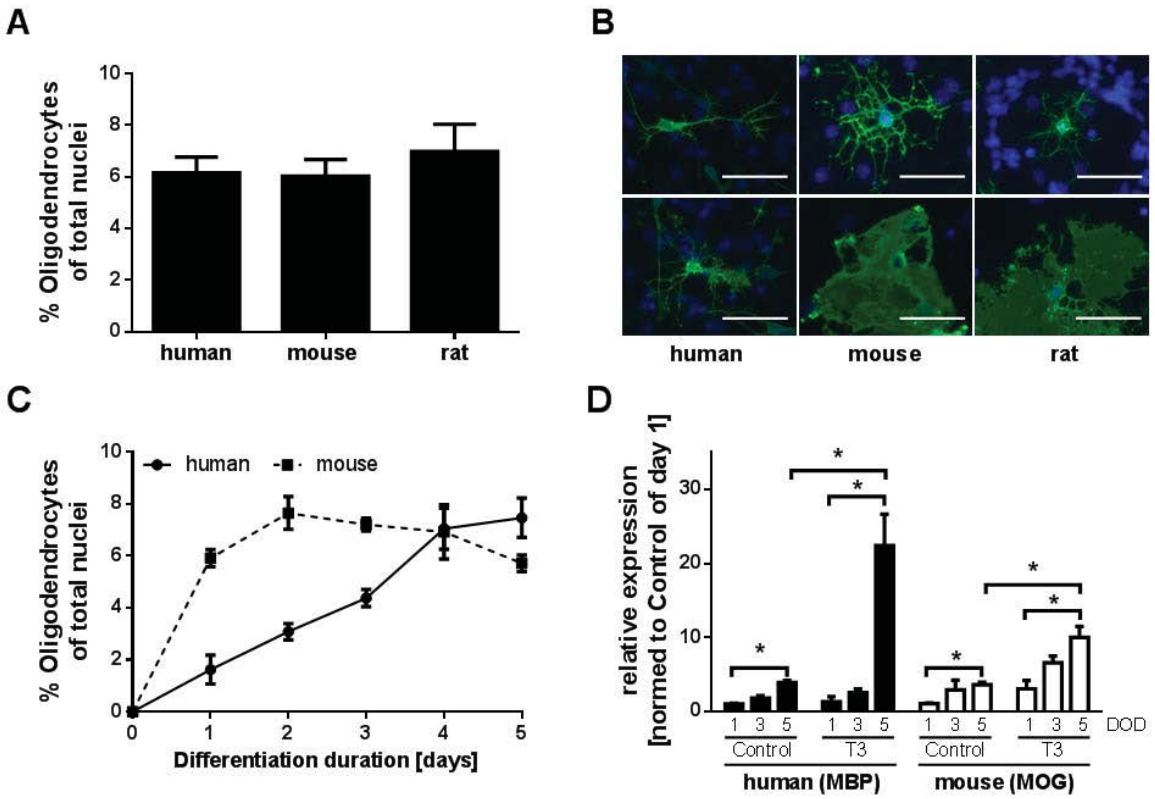

A
% Oligodendrocytes of total nuclei
human
mouse
rat
B
human
mouse
rat
C
% Oligodendrocytes of total nuclei
human
mouse
Differentiation duration [days]
D
relative expression [normed to Control of day 1]
DOD
Control
T3
human (MBP)
mouse (MOG)

Figure 13:

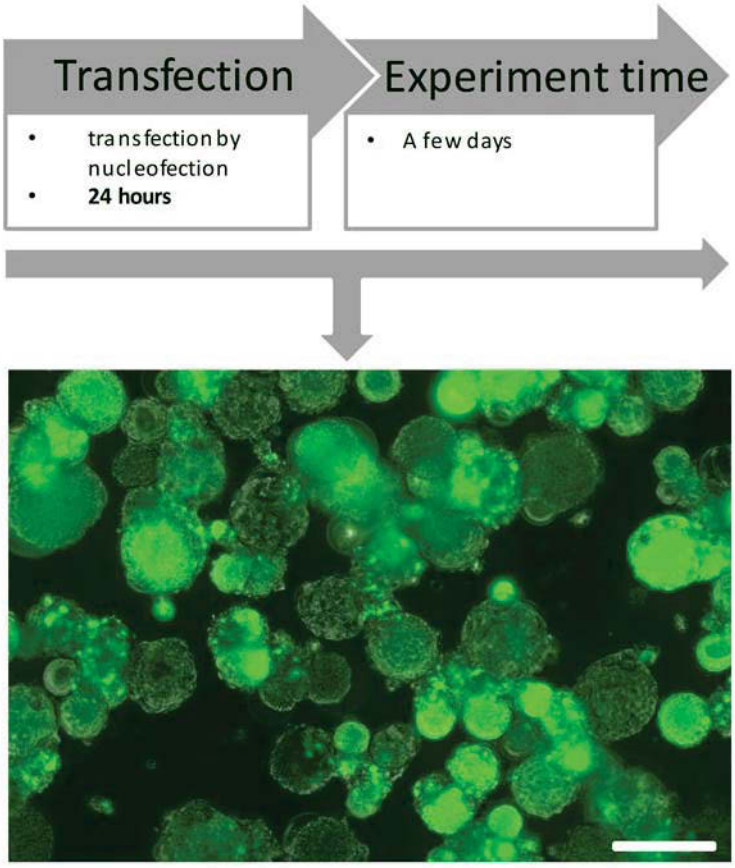

Transfection
transfection by nucleofection
24 hours
Experiment time
A few days

Figure 14:

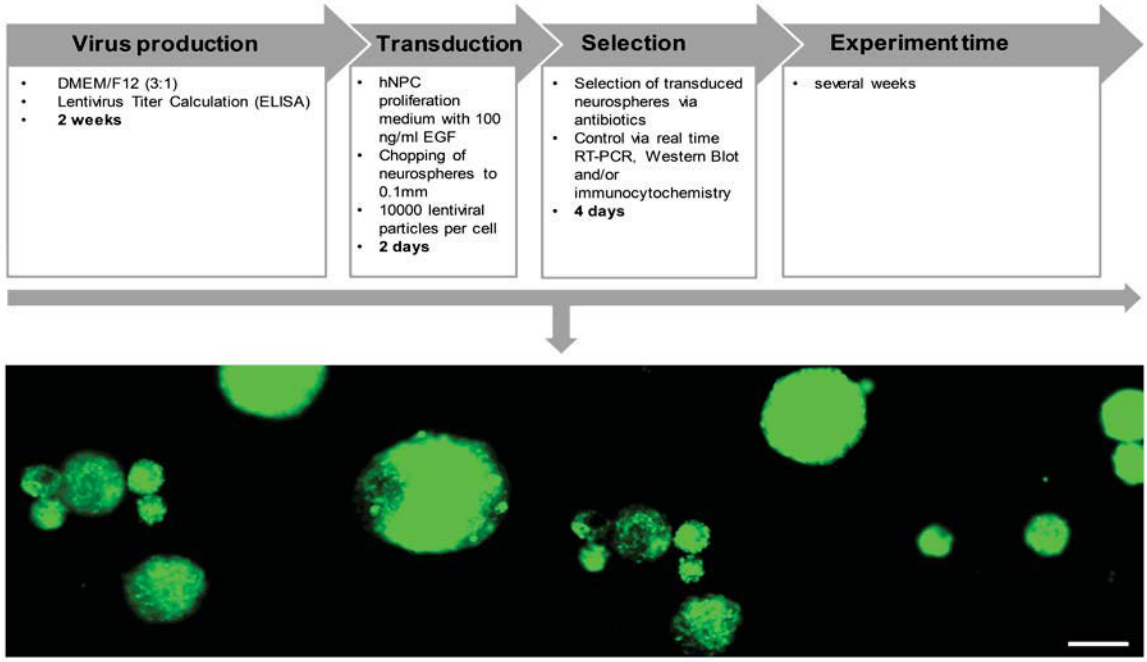
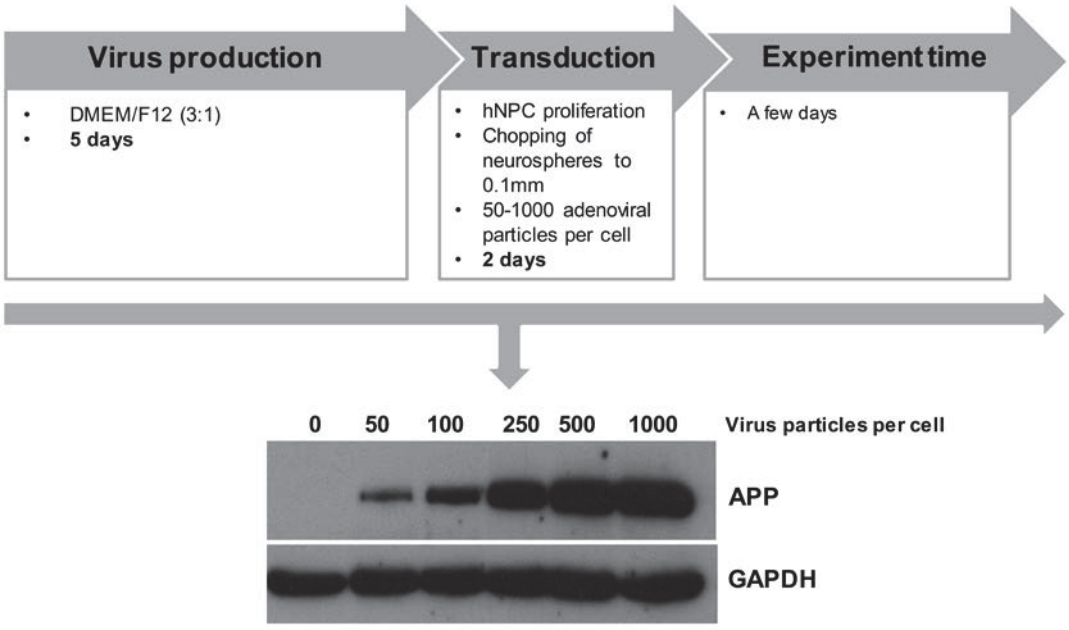

Virus production
DMEM/F12 (3:1)
Lentivirus Titer Calculation (ELISA)
2 weeks
Transduction
hNPC proliferation medium with 100 ng/ml EGF
Chopping of neurospheres to 0.1mm
10000 lentiviral particles per cell
2 days
Selection
Selection of transduced neurospheres via antibiotics
Control via real time RT-PCR, Western Blot and/or immunocytochemistry
4 days
Experiment time
several weeks

Figure 15:


Virus production
DMEM/F12 (3:1)
5 days
Transduction
hNPC proliferation
Chopping of neurospheres to 0.1mm
50-1000 adenoviral particles per cell
2 days
Experiment time
A few days
0
50
100
250
500
1000
Virus particles per cell
APP
GAPDH

Application of the Neurosphere Assay for DNT Hazard Assessment: Challenges and Limitations

J. Baumann, K. Dach, M. Barenys, S. Giersiefer, J. Goniwiecha, P. Lein, E. Fritsche

Name des Journals:	**Mechanisms and Predictive Modeling with *In vitro* and *In vivo* Approaches (Buchbeitrag)**
Impact Factor:	**wird nicht berechnet für Buchbeiträge**
Anteil an der Arbeit:	**50%** **Planung, Verfassen und Korrektur des Manuskripts**
Art der Autorenschaft:	**Erstautorenschaft**
Stand der Veröffentlichung:	**Eingereicht am 09. Januar 2015**

2.4. Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events

J. Baumann, K. Gassmann, S. Masjosthusmann, D. DeBoer, F. Bendt, S. Giersiefer, E. Fritsche

Archives of Toxicology [eingereicht am 1. April 2015]

Das sich entwickelnde Gehirn ist besonders anfällig gegenüber den adversen Effekten von Chemikalien, was im Menschen zu Entwicklungsstörungen des Nervensystems führen kann. Derzeit bilden Tierversuchsstudien in der Ratte den Goldstandard für die toxikologische Testung auf Entwicklungsneurotoxizität, allerdings sind diese Richtlinien-konformen Studien unzureichend bezüglich ihres Tierverbrauchs sowie Zeit- und Kostenbedarfs. Des Weiteren bergen Speziesunterschiede zum Mensch das Problem der Extrapolation. Aus diesem Grund besteht internationaler Konsensus über den Bedarf für die Entwicklung von Alternativmethoden, die das entwicklungsneurotoxische Potential von Chemikalien schneller, kostengünstiger und mit einer hohen Prädiktivität für den Menschen ermitteln können. In diesem Zusammenhang haben wir ein *in vitro* Model für das Screenen auf entwicklungsneurotoxische Schlüsselereignisse entwickelt, welches auf primären neuralen Progenitorzellen von Mensch und Ratte basiert, die als Neurosphären wachsen. Diese sind in der Lage, basale Prozesse der frühen fetalen Phase der Gehirnentwicklung nachzustellen und ermöglichen eine Untersuchung von Speziesunterschieden zwischen Mensch und Nager in korrespondierenden Zellmodellen. Ziel dieser Studie war es, zu untersuchen, in wie weit humane und Rattenneurosphären das entwicklungsneurotoxische Potential eines gut charakterisierten Chemikalien-Trainingsets mit neun Chemikalien richtig vorhersagen können, in dem Effekte auf die Endpunkte Progenitorzell-Proliferation, Migration und neuronale Differenzierung parallel zu Effekten auf die Viabilität untersucht wurden. Außerdem wurden die Chemikalieneffekte in humanen und Rattenneurosphären miteinander verglichen. Unsere Ergebnisse zeigen, dass (i) eine Korrelation unserer humanen und Rattendaten *in vitro* mit existierenden *in vivo* Daten für die meisten Chemikalien eine korrekte Vorhersage des entwicklungsneurotoxischen Potentials ermöglichte und humane und Rattenneurosphären dementsprechend eine wertvolle Komponente in einer modularen Testbatterie für Entwicklungsneurotoxizität bilden können, und (ii) humane und Rattenneurosphären sich in ihrer Empfindlichkeit gegenüber den meisten Chemikalien unterschieden haben und somit toxikodynamische Speziesunterschiede von Chemikalien widerspiegeln.

Archives of Toxicology

Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events

--Manuscript Draft--

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Abstract:	The developing brain is highly vulnerable to the adverse effects of chemicals resulting in neurodevelopmental disorders in humans. Currently, animal experiments in the rat are the gold standard for developmental neurotoxicity (DNT) testing, however, these guideline studies are insufficient in terms of animal use, time and costs and bear the issue of species extrapolation. Therefore, the necessity for alternative methods that predict DNT of chemicals faster, cheaper and with a high predictivity for humans is internationally agreed on. In this respect we developed an in vitro model for DNT key event screening, which is based on primary human and rat neural progenitor cells grown as neurospheres. They are able to mimic basic processes of early fetal brain development and enable an investigation of species differences between humans and rodents in corresponding cellular models. The goal of this study was to investigate to what extent human and rat neurospheres were able to correctly predict the DNT potential of a well-characterized training set of nine chemicals by investigating effects on progenitor cell proliferation, migration and neuronal differentiation in parallel to cell viability, and to compare these chemical responses between human and rat neurospheres. We demonstrate that (i) by correlating these human and rat in vitro results to existing in vivo data, human and rat neurospheres classified most compounds correctly and thus may serve as a valuable component of a modular DNT testing strategy and (ii) human and rat neurospheres differed in their sensitivity to most chemicals, reflecting toxicodynamic species differences of chemicals.
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Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events

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Abstract

The developing brain is highly vulnerable to the adverse effects of chemicals resulting in neurodevelopmental disorders in humans. Currently, animal experiments in the rat are the gold standard for developmental neurotoxicity (DNT) testing, however, these guideline studies are insufficient in terms of animal use, time and costs and bear the issue of species extrapolation. Therefore, the necessity for alternative methods that predict DNT of chemicals faster, cheaper and with a high predictivity for humans is internationally agreed on. In this respect we developed an *in vitro* model for DNT key event screening, which is based on primary human and rat neural progenitor cells grown as neurospheres. They are able to mimic basic processes of early fetal brain development and enable an investigation of species differences between humans and rodents in corresponding cellular models. The goal of this study was to investigate to what extent human and rat neurospheres were able to correctly predict the DNT potential of a well-characterized training set of nine chemicals by investigating effects on progenitor cell proliferation, migration and neuronal differentiation in parallel to cell viability, and to compare these chemical responses between human and rat neurospheres. We demonstrate that (i) by correlating these human and rat *in vitro* results to existing *in vivo* data, human and rat neurospheres classified most compounds correctly and thus may serve as a valuable component of a modular DNT testing strategy and (ii) human and rat neurospheres differed in their sensitivity to most chemicals, reflecting toxicodynamic species differences of chemicals.

Keywords

Neurosphere, Human, Rat, Developmental Neurotoxicity, *in vitro*, species difference

Introduction

The socio-economic potential of a population is substantially determined by the intelligence of its individuals (Bellanger et al. 2013). Therefore, it is of utmost importance to ensure individual development of maximum intellectual potential. Poisoning disasters with e.g. polychlorinated biphenyls or mercury have strikingly demonstrated that the developing brain is highly vulnerable to the adverse effects of chemicals (Rodier 1995) resulting in neurodevelopmental disorders in humans (Grandjean and Landrigan 2006). Not only poisoning incidences but also low-dose exposures towards environmental chemicals are thought to interfere with human brain development (Grandjean and Landrigan 2014) thus entailing a serious threat to society (Goldman and Koduru 2000). Currently, the rat bioassay is the gold standard for developmental neurotoxicity (DNT) testing (testing guidelines OECD TG426 and US-EPA 870.6300: OECD 2007; USEPA 1998) However, these guideline studies are resource-intensive (animals, time, money), bear the issue of species extrapolation and do not necessarily produce satisfying results (Coecke et al. 2007; Lein et al. 2007; Lein et al. 2005). Considering that the majority of chemicals on the market has not been studied for their DNT potential (Grandjean and Landrigan 2006), necessity for alternative methods, that predict DNT of chemicals faster, cheaper and with a high predictivity for humans, was recently agreed on by different stakeholders from regulatory agencies, industry and academia on both sides of the Atlantic (Bal-Price et al. 2015). Such alternative methods might also be used to assess DNT hazard in a mechanistic context of human relevance (Crofton et al. 2011).

To date, there are no validated alternative *in vitro* DNT assays available, but within the last years significant effort has been made to develop cell-based testing strategies for DNT hazard characterization of toxicants (Bal-Price et al. 2012; Breier et al. 2010; Coecke et al. 2007; Crofton et al. 2011; Lein et al. 2007; Lein et al. 2005). In parallel, toxicological testing principles have been subjected to a paradigm shift, proposing that chemical testing should move towards higher throughput, mechanism-oriented, preferably human-based methods to circumvent species-specific effects in responses to compound exposure (Krewski et al. 2010; NRC 2007; Seidle and Stephens 2009). Emphasis on the human nature of cell-based assays is a result of mainly pharmacological research with poor translation of drug candidates from highly cited animal research into clinical application (Leist and Hartung 2013). A prerequisite for human *in vitro* assay validation are known human toxicants. In the case of DNT, however, these are not given (Grandjean and Landrigan 2006; Grandjean and Landrigan 2014) and thus rodent *in vitro* testing systems provide valuable tools for studying assay performance which can then be translated to human systems.

In this respect we developed an *in vitro* model for DNT key event screening, which is based on primary human and rat neural progenitor cells grown as neurospheres (Baumann et al. 2014). They are able to mimic basic processes of early fetal brain development such as proliferation, migration and differentiation to neural effector cells (Fig. 1) and enable an investigation of species differences between humans and rodents in corresponding cellular models (Gassmann et al. 2010; Moors et al. 2007; Moors et al. 2009). Therefore, in the current study we investigated to what

extend human and rat neurospheres were able to correctly predict the DNT potential of a well-characterized training set of nine chemicals (Suppl. Table 1) and compared these chemical responses between human and rat neurospheres. By correlating these human and rat *in vitro* results to existing *in vivo* data we demonstrate that, depending on the biological application domain, human and rat neurospheres may serve as a valuable component of a modular DNT testing strategy.

Materials and Methods

***Cell culture*.** Normal human neural progenitor cells (hNPCs, male, GW 16-19) were purchased from Lonza Verviers SPRL (Verviers, Belgium). Rat neural progenitor cells (rNPCs, postnatal day (PND) 5) were prepared time-matched to hNPCs (Clancy et al. 2007) as described previously (Baumann et al. 2014).
Both human and rat NPCs were cultured in proliferation medium. Differentiation was initiated by growth factor withdrawal in differentiation medium and plating onto poly-D-lysine (PDL)/laminin–coated chamber slides as described previously (Baumann et al. 2014). For details, see Supplementary Material.

Cell viability assay. In every experiment mitochondrial reductase activity was assessed in the same wells than the specific endpoint evaluations as previously described (Baumann et al. 2014). For details, see Supplementary Material.

Cytotoxicity assay. For the cytotoxicity measurement the lactate dehydrogenase (LDH) assay (CytoTox-One; Promega, Mannheim, Germany) was used as described previously (Baumann et al. 2014). For details, see Supplementary Material.

Proliferation analysis. NPC proliferation was measured by the Cell Proliferation ELISA, BrdU (chemiluminescent) from Roche (Mannheim, Germany) as described previously (Baumann et al. 2014). Spheres cultivated in proliferation medium without growth factors served as endpoint specific control and for correction of unspecific binding of the BrdU antibody some spheres were cultured without BrdU.

Migration analysis. Migration analyses were performed as previously described (Baumann et al. 2014). 10 µM PP2 (Sigma Aldrich, Taufkirchen, Germany), a selective inhibitor for Src-family kinases, was used as endpoint specific control (Moors et al. 2007).

Differentiation analysis. Differentiated spheres were fixed in 4% paraformaldehyde for 30 min at 37° C. Neurons were identified by immunocytochemical staining against β(III)-tubulin and

quantified as previously described (Baumann et al. 2014). As endpoint specific control spheres were cultured in differentiation medium with 20 ng/ml epidermal growth factor (EGF; Ayuso-Sacido et al. 2010).

Chemical Preparation and Exposure. A set of 9 commercially available test chemicals was chosen to develop a protocol for screening chemicals over a wide concentration range (Suppl. Table 1). Six chemicals were selected based on data demonstrating adverse effects on the developing nervous system (positive substances). Another three chemicals were selected based on the presumed absence of data indicating effects on the developing nervous system (negative substances). For further information on chemicals, see Supplementary Material. For each experiment, stock solutions were diluted according to their starting concentration in medium (Suppl. Table 1) and serial 1:3 dilutions were prepared from this starting concentration in medium with the respective solvent concentration.

Under proliferative conditions, human and rat neurospheres were plated one sphere per well into 96-well plates in 100 µl of exposure media (proliferation medium + test compound). Four wells per exposure condition were used to assess proliferation by BrdU incorporation as well as viability. To measure cell migration or differentiation in combination with viability, five neurospheres were plated in one well of a PDL/laminin coated 8-chamber-slide under differentiating conditions. For assessment of migration, cells were exposed to chemicals for 24 hrs and for proliferation or differentiation analyses, the exposure duration was 72 hrs (Fig. 1). Each experiment was repeated at least three times on separate days and with different preparations of rat neurospheres or in case of the human neurospheres with cells of 2-3 different donors.

Statistics. Data analysis was performed using Graph-Pad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). In concentration response experiments, all data were normalized to the respective solvent control and are presented as mean percent of solvent control ± standard error of the mean (SEM). Chemical effects were determined using a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Data obtained at each chemical concentration were compared to respective vehicle control, $p \leq 0.05$ was considered significant. For the sigmoidal dose response curve fitting and the calculation of the EC_{50} values and 95 % confidence intervals, a four-parameter logistic nonlinear regression model with the top set to 100 % and the bottom set to 0 % was used. Data were collected across 3 to 15 independent experiments with four to five neurospheres each. For pairwise comparisons, student's t-test was performed with $p \leq 0.05$ considered as significant.

Results

For chemical testing in human and rat NPCs we developed a testing scheme in which neurospheres were mechanically dissociated by chopping three days prior to plating in order to

obtain a defined and uniform sphere population. Under proliferative conditions, floating neurospheres were exposed to testing chemicals for three days and afterwards assessed for changes in proliferation and viability. Under differentiating conditions, neurospheres plated on laminin-coated surfaces were exposed to testing chemicals for 24 hrs to assess migration by measuring migration distances and viability, and for evaluating neuronal differentiation, spheres were exposed for three days to analyze the neuronal marker βIII-tubulin and viability (Fig. 1). This experimental setup allows (i) a distinction of specific chemical effects on neurodevelopmental endpoints and viability and (ii) a direct comparison of such between human and rat NPCs.

The usage of endpoint specific controls is one important criterion for the development of alternative methods for chemical screening (Crofton et al. 2011). Therefore, we established control chemicals which reliably change the respective endpoint to a certain amount without reducing viability. Proliferation was inhibited by growth factor withdrawal, which reduced BrdU luminescence from 190238 ± 20102 RLU to 50673 ± 18312 RLU in hNPCs, and from 64534 ± 13155 RLU to 23307 ± 6242 RLU in rNPCs (Suppl. Fig. 1a), respectively, whereas no cytotoxicity was detected by LDH assay (Suppl. Fig. 1b). The src-kinase inhibitor PP2 reduced migration distances (Moors et al. 2007) in hNPCs from 404 ± 12 to 247 ± 12 µm 24 hrs after plating, and from 456 ± 39 to 73 ± 18 µm in rNPCs, respectively (Suppl. Fig. 1c). Again, viability was not reduced (hNPCs) or reduced to a lesser extent than migration (rNPCs, Suppl. Fig. 1d). EGF was used to inhibit neuronal differentiation (hNPCs: 9.6 ± 0.6 to 1.9 ± 0.3 % neurons; rNPCs: 15.2 ± 1.6 to 0.6 ± 0.3 % neurons) without being cytotoxic (Suppl. Fig. 1e and f).

Next, we tested a training set of six positive and three negative compounds (Suppl. Table 1) for their effects on proliferation, migration and neuronal differentiation in human and rat NPCs (Fig. 2 and Suppl. Fig. 2-4). For every endpoint and chemical, concentration response curves were recorded and EC_{50} values with their corresponding 95% confidence intervals were calculated after performing a sigmoidal dose response curve fitting (Table 1). Because we assume that disturbance of any neurodevelopmental key event will cause an adverse neurodevelopmental outcome, the most sensitive endpoint (MSE) for every chemical and species was determined and compared to its corresponding EC_{50} value for viability (Fig. 3a) to decide if specific effects on proliferation, migration or neuronal differentiation can be distinguished from general cytotoxicity (Crofton et al. 2011). Moreover, the EC_{50} values for the MSE for each compound within each species regardless of the nature of the endpoint determined the more sensitive species.

The MSE after NPC exposure towards MeHgCl was neuronal differentiation (hNPCs: 56.22 nM; rNPCs: 29.55 nM); with viability affected in both species at a higher order of magnitude (hNPCs: 815.7 nM; rNPCs: 234.6 nM; Fig. 2 and Table 1). Confidence intervals (95%) of EC_{50} values for the MSE and viability did not overlap in either rat or human NPCs showing that MeHgCl specifically inhibited neuronal differentiation. Moreover, 95% confidence intervals for the MSE in human and rat NPCs did not overlap either, demonstrating the higher sensitivity of rat versus human NPCs towards MeHgCl exposure.

Upon $NaAsO_2$ treatment hNPC proliferation was the MSE (EC_{50} = 1.728 µM; Suppl. Fig. 2 and Table 1), whereas neuronal differentiation was inhibited most potently in rNPC (EC_{50} = 0.4061 µM, Suppl. Fig. 4 and Table 1). EC_{50} values for viability were either higher than the MSE (human: 4.574 µM) or not reached at all (rat; Suppl. Fig. 2 and 4, and Table 1) supporting specific DNT effects of $NaAsO_2$. However, with regards to the respective MSE, rNPCs were more sensitive than hNPCs.

The EC_{50} value for chlorpyrifos was only reached for the endpoint neuronal differentiation in hNPCs, although the curve for viability was mostly overlapping (140.5 µM; Suppl. Fig. 4 and Table 1). In contrast, proliferation was the MSE in rNPCs (28.54 µM; Suppl. Fig. 2 and Table 1) with the EC_{50} value for viability not reached. Thus, within the endpoints studied, rNPCs were the more sensitive species towards chlorpyrifos.

Parathion only impaired the endpoint neuronal differentiation in hNPCs (252.5 µM) and viability under differentiating conditions in rNPCs (251.2 µM; Suppl. Fig. 4 and Table 1). Looking at the concentration response curves for hNPCs, it is likely that parathion did specifically impair neuronal differentiation and although the EC_{50} value for the endpoint migration was not reached, the highest concentration tested (257 µM) significantly reduced migration (Suppl. Fig. 3).

MAM inhibited both proliferation and neuronal differentiation in human and rat NPCs at similar potencies. However, proliferation was chosen as MSE (human EC_{50} = 325.7 µM, rat EC_{50} = 31.82 µM) as effects between proliferation and viability deviated most for both species (human EC_{50} = 1245 µM, rat EC_{50} = 247 µM; Fig. 2 and Table 1). Rat NPCs were found to be more vulnerable towards MAM-induced reduction in proliferation than hNPCs.

hNPC proliferation was specifically inhibited by NaVPA (EC_{50} = 756.3 µM, Suppl. Fig. 2 and Table 1) without affecting viability (EC_{50} not reached within tested concentration range). In contrast, NaVPA reduced proliferation and neuronal differentiation in rNPCs at similar concentrations (EC_{50} = 379.5 µM and EC_{50} = 321.1 µM, respectively) distinguishable from effects on viability (EC_{50} = 4019 µM and EC_{50} = 1903 µM, respectively; Suppl. Fig. 2 and 4, Table 1). The MSE of rNPCs was more sensitive than hNPCs.

Exposure to sodium glutamate revealed an inhibition of proliferation as only specifically affected endpoint in hNPCs with an EC_{50} value of 1938 µM (Suppl. Fig. 2 and Table 1). In rNPCs, neuronal differentiation was specifically inhibited at lower concentrations (EC_{50} = 374.6 µM, viability: EC_{50} = 8655 µM; Suppl. Fig. 4 and Table 1), making the rat again the more sensitive species.

Paracetamol specifically inhibited proliferation in hNPCs (EC_{50} = 2219 µM, viability: EC_{50} = 3884 µM; Suppl. Fig. 2 and Table 1). rNPCs were more sensitive than human ones, and the endpoint neuronal differentiation was most sensitive and specifically inhibited in the rat (EC_{50} = 399.1 µM, viability: EC_{50} = 1538 µM; Suppl. Fig. 4 and Table 1).

Last, penicillin G only had a specific effect on proliferation in hNPCs (EC_{50} = 2512 µM), whereas in rNPCs the EC_{50} value was not reached for any of the endpoints (Fig. 2 and Table 1) although the highest concentration (10000 µM) significantly reduced proliferation as well.

Discussion

During the last decade, when the toxicological paradigm shift towards more mechanism- and pathway-driven approaches for human hazard and risk assessment has been evolving, also alternative assay development for DNT testing has gained priority within the regulatory environment (Bal-Price et al. 2015). This is mainly due to the enormous resource-intensity of the DNT guideline studies and their high variability supported by the over-all dissatisfactory prediction of animals to humans (Leist and Hartung 2013). As one approach to DNT *in vitro* testing, we developed a 3D cell culture model based on primary human and rat NPCs grown as neurospheres (Baumann et al. 2014; Moors et al. 2009). According to general recommendations for alternative methods development (Crofton et al. 2011), here we demonstrate that (i) the neurosphere assay can be used to determine concentration-response-effects of a training set of chemicals on key events of neurodevelopment (proliferation, migration and neuronal differentiation) in a species-specific manner (Fig. 2). (ii) By using endpoint-specific controls key events can reliably and consistently be modulated (Suppl. Fig. 1), (iii) this experimental setup enables a determination of the respective endpoint multiplexed with viability to distinguish specific chemical actions on neurodevelopmental key events from secondary effects due to cell death (Fig. 2 and 3) and (iv) data cannot be interpreted on a pure hazard basis but needs exposure data for correct chemical classification (Fig. 3).

Species differences entail an important issue for regulators in pharmacology and toxicology as the predictive value of animal experiments for effects in humans is often poor (Leist and Hartung 2013). By directly comparing chemical effects on neurodevelopmental key events of rat and human neurospheres generated from equivalent developmental time points (Clancy et al. 2007), species differences based on cellular toxicodynamics can be tackled. Our study shows that rat and human NPCs differ in their susceptibility to almost all of the chemicals tested. For this set of compounds, rNPCs respond over all at lower concentrations than hNPCs (Table 1 and Fig. 3a). As this compound set is rather small, no general conclusion can be drawn from these data on general species-specific sensitivity of NPCs from humans and rats. Testing of more compounds with different modes of action (MOA) is rather needed to get a more detailed view on pathway-specific sensitivities across these species. Moreover, this data set suggests that neuronal differentiation might be the MSE in rNPCs, while this seems to be NPC proliferation for hNPCs. This conclusion would also be premature due to the small number of compounds in this training set and more compound testing will reveal if at all such a general assumption can be made. Species differences in sensitivity towards DNT chemicals have sparsely been evaluated so far. Differences were found for compound-compromised neurite outgrowth in human ESC-derived neural cultures and rat cortical cultures (Harrill et al. 2011) as well as for chemically-induced reduction in NPC proliferation and migration in primary human versus mouse cultures (Gassmann et al. 2010). Given the fact that molecular equipment of the human developing brain seems to contain unique features in the animal kingdom (Somel et al. 2011; Zhang et al. 2011), it seems necessary to understand human-

specific developmental toxicity of compounds to this sensitive organ. Such information combined with MOA analyses of chemicals can provide information on molecular and functional differences between rodents and humans which can be applied in a quantitative way to determine if the animal data has any relevance to humans and if interspecies uncertainty factors need to be adjusted (Burgess-Herbert and Euling 2013).

One way of determining if these hazards are at all relevant to human health is implementation of exposure. We compared the experimentally assessed human and rat EC_{50} values from this study to *in vivo* internal exposure levels of the nine testing chemicals in humans and rats for a comparative risk assessment according to the parallelogram approach (Fig. 3b). Due to the lack of information on precise MOA of DNT compounds this comparative *in vitro - in vivo* approach is imperfect. E.g. the key event neurogenesis is hardly studied *in vivo*. Because neurogenesis was the most sensitive endpoint for many of the compounds tested in this training set in rat neurospheres, we chose data on cognitive *in vivo* endpoints as the adverse outcome (AO) if no other data was available and correlated AO LOAELs with EC_{50} values for functional endpoints studied *in vitro*. This highlights the need for more mechanistic data on DNT compounds for a comprehensive correlation of *in vivo* and *in vitro* effects.

Prenatal MeHgCl exposure causes mental retardation and developmental delays in children (Grandjean and Landrigan 2006). Neuropathological examinations showed microcephaly and global brain disorganization due to disturbances in cell migration and division (Schettler 2001). Likewise, hNPC proliferation and migration were specifically inhibited by MeHgCl *in vitro*, but the most sensitive endpoint was neuronal differentiation with an EC_{50} value of 56 nM. *In vivo* studies revealed that a maternal hair concentration of 4.5 ppm MeHgCl as the lowest observed adverse effect level (LOAEL) found in the literature results in neuropsychological deficits in children (Castoldi et al. 2001). According to toxicokinetic calculations (Burbacher et al. 1990; Lewandowski et al. 2003) this hair concentration should resemble an infant brain concentration of approximately 72 nM. In rats, prenatal low-dose administrations of 0.01 mg/kg MeHgCl from gestational day (GD) 6 to 9, which are estimated to result in maximal fetal brain concentrations of 30 nM (Burbacher et al. 1990; Lewandowski et al. 2003), affected learning behavior in the progeny (Bornhausen et al. 1980). Similarly, rNPC proliferation, migration and neuronal differentiation were affected at subcytotoxic concentrations, whereas neuronal differentiation was most sensitive (EC_{50} = 30 nM). Arranging experimentally obtained *in vitro* and calculated internal *in vivo* concentrations in a parallelogram demonstrates a good correlation between *in vitro* and *in vivo* concentrations for both species (Fig. 3b). A similar approach was carried out by Lewandowsky and coworkers (2003) who summarized that rat neuroblast proliferation *in vitro* and *in vivo* was inhibited at similar orders of magnitude (approx. 1 µM (Ponce et al. 1994) and 3 µM (Chen et al. 1979) MeHgCl, respectively). Our data for rNPC proliferation is in good agreement with these historical *in vitro* data (Table 1). However, proliferation was not the MSE for MeHgCl in this study and to the best of our knowledge effects of MeHgCl on neuronal differentiation *in vivo* has not been studied so far.

The pesticide chlorpyrifos was recently classified as a human developmental toxicant (Grandjean and Landrigan 2014). In hNPCs chlorpyrifos affected neuronal differentiation with an EC_{50} value of 141 µM in a rather non-specific way as concentration-response curves for neuronal differentiation and viability overlapped. In a prospective cohort study examining early childhood development after prenatal exposure to chlorpyrifos, altered attention was detected in highly exposed children. Cord blood concentrations with a LOAEL of 6.17 pg/g were measured (Rauh et al. 2006), translating to a concentration of 18 pM. Although children's brain concentrations were not calculated, it is obvious that the experimentally-derived results from hNPC *in vitro* are far from any *in vivo* relevance. In rNPCs proliferation was specifically inhibited with an EC_{50} value of 29 µM. Similarly, an administration of 1 mg/kg chlorpyrifos between PND 1 and 4 decreased DNA-synthesis in the brain (Dam et al. 1998). According to pharmacokinetic modeling, this dose would result in a brain concentration of 2.1 µM (Timchalk et al. 2006), which is around 10 times lower than the effective *in vitro* concentration inhibiting rat NPC proliferation. Thus, human and rat NPCs failed to predict the DNT potential of chlorpyrifos correctly as effects were not seen unless toxicologically irrelevant concentrations were applied. This could be due to lack of cytochrome P450 metabolism in developing brain cells (Gassmann et al. 2010; Jiang et al. 2010), chlorpyrifos acting on earlier phases of brain development, on later neurodevelopmental endpoints like axon and dendrite formation and synaptogenesis (Howard et al. 2005; Yang et al. 2008) or in an indirect way e.g. involving neuroinflammation, which cannot be assessed with this assay.

MAM disturbs central nervous system development during the fetal and neonatal period (Cattabeni and Di Luca 1997). It mainly acts through an inhibition of proliferation and affects developing neurons through DNA alkylation (Kisby et al. 2009). In line with this, the endpoints proliferation and neuronal differentiation were specifically inhibited in both human and rat NPCs at concentrations of 326-345 µM (human) and 23-32 µM (rat). Although developmental MAM exposure through contaminated cycad flour is strongly linked to neurological disorders in the western pacific (Spencer et al. 1991), there is no reliable data available on human exposure levels. However, in rats an administration of 7.5 mg/kg between GD 13 and 15 caused substantial changes in brain morphology (De Groot et al. 2005). According to a study of Bassanini and coworkers (2007) such a dose probably results in a fetal brain concentration of 30 µM which is very similar to the effective concentrations in our rat *in vitro* results (EC_{50} = 31.82 µM; Table 1), demonstrating that rNPCs were able to predict the actual risk of MAM properly.

The antibiotic penicillin G was used as a negative DNT compound in this study. Penicillin inhibited proliferation of only hNPCs at high concentrations (2512 µM). Therapeutic plasma and CSF concentrations are several orders of magnitude lower than the effective concentration measured in the hNPC *in vitro* system (111 µM and 2.4 µM, respectively; Karlsson et al. 1996). Thus this compound is classified correctly as a negative substance with regard to health risk. For further discussion of the remaining five test chemicals, see Supplementary Discussion.

Taking species-specific human and rat internal exposure levels into account, four out of six DNT positive compounds and all three negative compounds were classified correctly by assessing the four endpoints viability, NPC proliferation, migration and neuronal differentiation using human and rat NPCs (Fig. 3a). For the data rich compounds MeHgCl and NaVPA a comprehensive risk assessment according to the parallelogram approach was possible and revealed that for both species *in vivo* and *in vitro* concentrations correlated well with disturbance of neurodevelopmental endpoints *in vivo* (Supplementary discussion, Suppl. Fig. 5). This supports the hypothesis articulated earlier that neurodevelopmental processes as key events of brain development can be mimicked *in vitro* and might serve as the basis for alternative DNT testing strategies *in vitro* (Lein et al. 2005). For arsenic and MAM, only rat internal exposure concentrations were available so that a conclusive assessment of hNPC data was not feasible. Due to the good correlations of the available rat *in vivo* and *in vitro* data on those two compounds a correct classification of arsenic and MAM based on human NPC data is thus likely. This example demonstrates that human toxicokinetic modeling to estimate internal exposure levels has utmost importance for a comprehensive decision making process if *in vitro* results are implemented (Croom et al. 2015; Patlewicz et al. 2015).

In contrast to the correctly identified DNT compounds, the two pesticides chlorpyrifos and parathion were not correctly classified as DNT positive compounds in the human and rat neurosphere assay as EC_{50} values exceeded their estimated effective internal exposure levels. This might be due to the reasons discussed above. Specifically, chlorpyrifos seems to inhibit axonal growth and induce dendritic growth in primary rat neuronal cultures at nanomolar concentrations or below (Howard et al. 2005). This clearly indicates that it is very important to define the biological application domain of each *in vitro* system to determine which MOA it is able to assess and especially where its limitations lie. This knowledge is necessary to gain certainty about its use in a regulatory context (Bal-Price et al. 2015).

All three DNT negative compounds affected NPC development at toxicologically absolutely irrelevant concentrations, demonstrating that human and rat NPCs were able to detect negative compounds correctly.

One reason why appropriate test concentrations are of high importance in such physiologically-relevant organoids consisting of primary cells might be the correct homeostasis of cellular components in these cells resembling the *in vivo* situation. Two notions support this assumption. For one, *ex vivo* NPCs seem to maintain their properties after taking them out of the whole organism, which was shown by compound effects in *in vivo*-*ex vivo* comparisons (Foti et al. 2013; Go et al. 2012; L'Episcopo et al. 2013). Secondly, the 3D format of cultures with cell-cell communication and interaction supports physiological cellular functions and thus *in vivo*-relevant responses towards xenobiotics (Alépée et al. 2014; Yamada and Cukierman 2007). Thus, we expected neurospheres to react only at compound concentrations relevant for interfering with signaling pathways necessary for the tested endpoints. That such a physiological context of

primary cells has a strong implication on *in vitro* testing has recently also been shown by Kleinstreuer and coworkers (2014). In this very elegant work the authors did not identify VPA as an HDAC inhibitor and they discussed that this is probably due to insufficient test concentrations of this drug (40 µM), which is pharmacologically active in the mM range.

In summary, results of a training set of nine chemicals in human and rat NPCs revealed that species differed in their sensitivity to most chemicals. A comparison of rat and human *in vivo* internal exposure levels and *in vitro* results seem to correlate well for compounds where data is available. Due to insufficient information, however, such a comparison could not be made for all compounds. In combination with assays that have the ability to assess chemical effects on early neurodevelopment and methods evaluating further key events needed for proper neuronal network formation (e.g. axon, dendrite, spine, synapse formation, neuronal network activity) the neurosphere assay is a valuable tool for DNT testing (Fig. 4). Because we have previously shown that the throughput of our assay can be increased by automation of neurosphere sorting and plating (Gassmann et al. 2012), this method renders useful for medium throughput applications. High content image analyses methods are on the way to further facilitate evaluation of such complex, multi-cellular structures. Data from such testing strategies can then be integrated into the 'Adverse Outcome Pathway' (AOP) framework (Ankley et al. 2010) and will help to develop so called 'Integrated Approaches to Testing Assessment' (IATA), which gather and weigh any existing relevant information – *in vivo*, *in vitro*, *in silico* and *in chemico* – to support regulatory or safety decisions (Tollefsen et al. 2014).

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Ethical Statement:

The animals used for NPC preparation were maintained in an accredited on-site testing facility according to the guideline provided by the Society for Laboratory Animals Science (GV-SOLAS). They were treated humanely and with regard for alleviation of suffering. NPC preparation was approved by the North Rhine-Westphalia State Environment Agency.

Conflict of Interest:

The authors declare that they have no conflict of interest.

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Tables:

Table 1: EC_{50} values and 95% confidence intervals for the nine compounds tested in human and rat neurospheres. Values are given in micromolar concentrations. The lowest obtained EC_{50} value for each species and compound is marked in red

	BrdU		Alamar Blue (PROL)		Migration Distance		Alamar Blue (MIG)		Neuronal Differentiation		Alamar Blue (DIFF)	
	human	rat	human	rat	human	rat	human	rat	human	rat	human	rat
MeHgCl												
EC_{50}	0.7046	0.1301	1.134	1.678	0.6466	1.543	> 3	> 3	0.05622	0.02955	0.8157	0.2346
95% CI	0.5973 to 0.8310	0.09460 to 0.1789	0.7434 to 1.730	1.200 to 2.345	0.5070 to 0.8245	1.404 to 1.696			0.04673 to 0.06762	0.02271 to 0.03847	0.7149 to 0.9307	0.2039 to 0.2701
AsNaO2												
EC_{50}	1.728	4.067	4.574	> 10	5.749	> 10	> 10	> 10	3.203	0.4061	7.360	> 10
95% CI	1.167 to 2.560	3.453 to 4.791	3.469 to 6.032		4.480 to 7.377				2.515 to 4.079	0.2860 to 0.5766	6.942 to 7.803	
CPF												
EC_{50}	> 213.9	28.54	> 213.9	> 213.9	> 213.9	> 213.9	> 213.9	> 213.9	140.5	48.56	> 213.9	> 213.9
95% CI		20.44 to 39.83							112.7 to 175.2	30.49 to 77.32		
Parathion												
EC_{50}	> 257.5	> 257.5	> 257.5	> 257.5	> 257.5	> 257.5	> 257.5	> 257.5	252.5	> 257.5	> 257.5	251.2
95% CI												
MAM												
EC_{50}	325.7	31.82	1245	247	> 1500	803.6	> 1500	> 1500	344.5	23.47	648.9	82.75
95% CI	277.3 to 382.5	26.93 to 37.61	1145 to 1353	192.7 to 316.5		706.5 to 914.2			297.7 to 398.7	19.11 to 28.82	606.5 to 694.3	66.61 to 102.8
NaVPA												
EC_{50}	756.3	379.5	> 11250	4019	> 11250	7625	> 11250	9729	3177	321.1	2399	1903
95% CI	547.5 to 1045	356.7 to 403.8		3811 to 4238		5911 to 9836		6597 to 14349	2009 to 5026	252.2 to 408.8	1911 to 3011	1390 to 2606
Glutamate												
EC_{50}	1938	5122	> 10000	> 10000	> 10000	7663	> 10000	> 10000	> 10000	374.6	> 10000	8655
95% CI	1514 to 2479	3894 to 6737				4883 to 12025				222.3 to 631.3		5535 to 13532
Paracetamol												
EC_{50}	2219	790.9	3884	4113	> 5000	> 5000	3693	> 5000	> 5000	399.1	3617	1538
95% CI	1859 to 2648	686.4 to 911.2	3370 to 4476	3516 to 4812			3118 to 4373			285.1 to 558.6	2708 to 4832	1073 to 2205
PenG												
EC_{50}	2512	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000
95% CI	1598 to 3948											

Figure Legends:

Fig. 1 Schematic overview of the experimental setup and chemical treatment periods of human and rat neurospheres. Human and rat neurospheres are exposed to test compounds (indicated in red) as floating neurospheres for assessing proliferation (day 0-3) or as plated neurospheres to assess either migration (day 0-1) or neuronal differentiation (day 0-3). For all endpoints, viability is investigated in parallel. Time line is in days. Scale bars **a and b** 300 µm, **c** 100 µm. **c.** Red: GFAP positive cells, green: βIII-tubulin positive cells, blue: cell nuclei

Fig. 2 Representative concentration-response curves for the endpoints proliferation, migration and neuronal differentiation. Concentration-response curves for three representative testing compounds in human (**a-i**) and rat neurospheres (**j-r**) are shown**. a-c and j-l:** Proliferation, **d-f and m-o:** migration, **g-i and p-r:** neuronal differentiation. **a, d, g, j, m, p:** MeHgCl; **b, e, h, k, n, q:** MAM; **c, f, i, l, o, r:** PenG. Values are given as average percentages of solvent control for the endpoints proliferation (BrdU), migration (mig. dist.) and neuronal differentiation (neuronal diff.) and the respective viability data (Alamar Blue) ± SEM (n = 3-8 independent experiments). Asterisks denote significance respect to solvent control for the endpoint proliferation/migration/neuronal differentiation and crosses denote significance respect to solvent control for the endpoint viability ($p < 0.05$). For curves of the remaining six testing compounds and experimental details, see Supplementary Fig. 2-4

Fig. 3 Pairwise comparison of the most sensitive endpoint and viability between human and rat neurospheres. **a.** EC_{50} values of the most sensitive endpoint (MSE) and viability in human and rat neurospheres for each testing compound are shown with its 95% confidence intervals and, if available, internal exposure levels of humans and rats. MSEs and estimated or measured internal exposures are as follows: MeHgCl – neuronal differentiation (hNPCs and rNPCs), brain concentration (hNPCs and rNPCs); $NaAsO_2$ – proliferation (hNPCs) and neuronal differentiation (rNPCs), estimated brain concentration (rNPCs); Chlorpyrifos - neuronal differentiation (hNPCs) and proliferation (rNPCs), brain concentration (rNPCs); Parathion - neuronal differentiation (hNPCs), brain concentration (rNPCs); MAM – proliferation (hNPCs and rNPCs), brain concentration (rNPCs); NaVPA – proliferation (hNPCs) and neuronal differentiation (rNPCs), brain concentration (hNPCs and rNPCs); Glutamate – proliferation (hNPCs) and neuronal differentiation (rNPCs), plasma level (hNPCs) and brain concentration (rNPCs); Paracetamol – proliferation (hNPCs) and neuronal differentiation (rNPCs), CSF concentration (hNPCs and rNPCs); PenG – proliferation (hNPCs) and CSF concentration (hNPCs). n.r. = EC_{50} not reached within the tested concentration range **b.** EC_{50} values of MeHgCl for MSEs in human and rat neurospheres are applied in a parallelogram approach. Therefore, existing rat *in vivo* data are compared to rat *in vitro* data to illustrate *in vivo* - *in vitro* similarities/differences. Rat *in vitro* data are compared with human *in vitro* data to obtain information regarding interspecies differences. All these data will then allow

an extrapolation of possible effects in humans *in vivo*. Green = experimental data, red = extrapolation

Fig. 4 Testing strategy for *in vitro* DNT testing. The assessment of different early and late neurodevelopmental key events provides a comprehensive approach for developmental neurotoxicity testing. Thereby, the endpoints evaluated within the Neurosphere Assay integrate into early fetal development. ESC = embryonic stem cell, NCC = neural crest cell, NEP = neuroepthelial precursor cell, NS/PC = neural stem/progenitor cell

Figure 1

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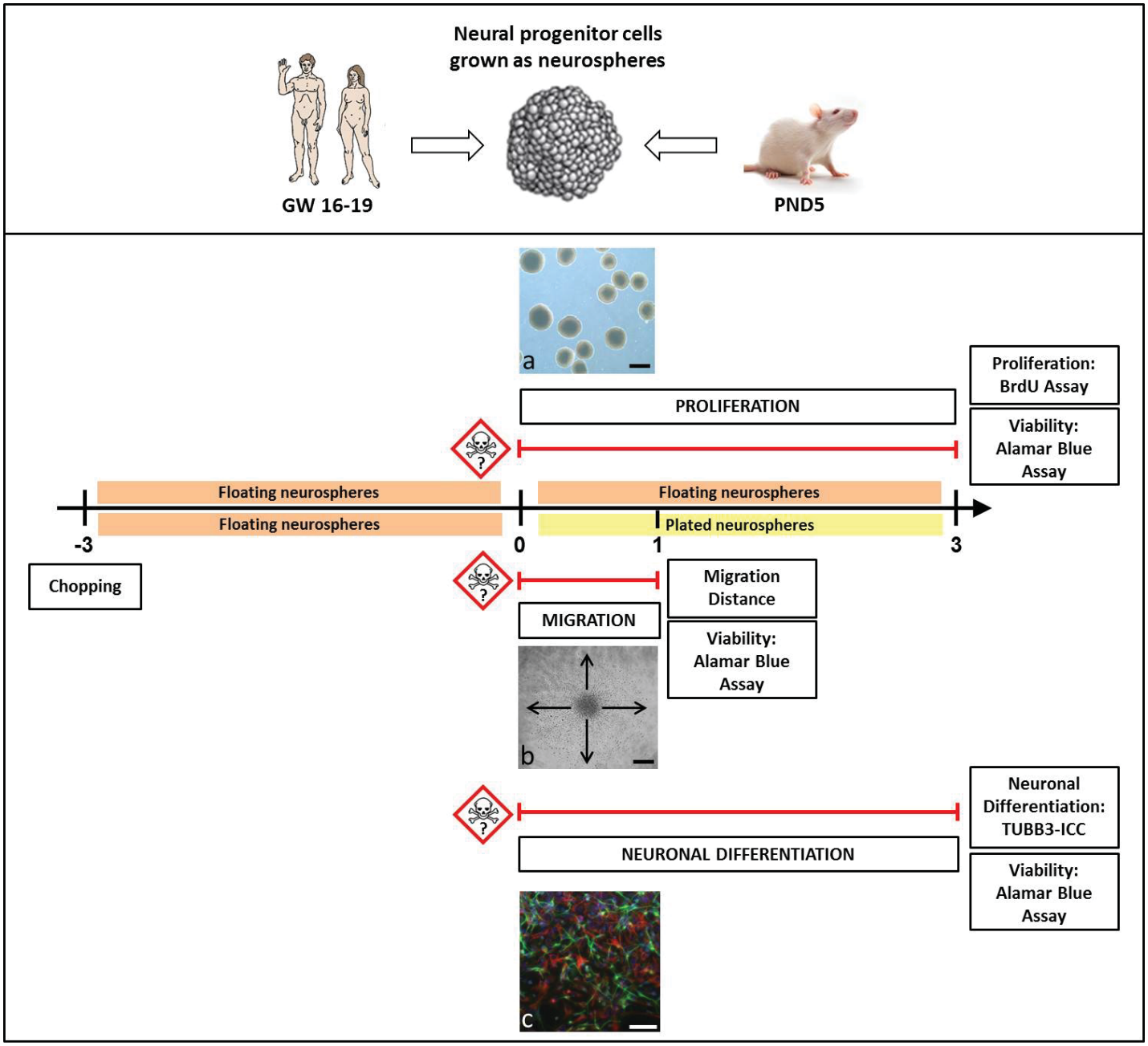

Figure 2

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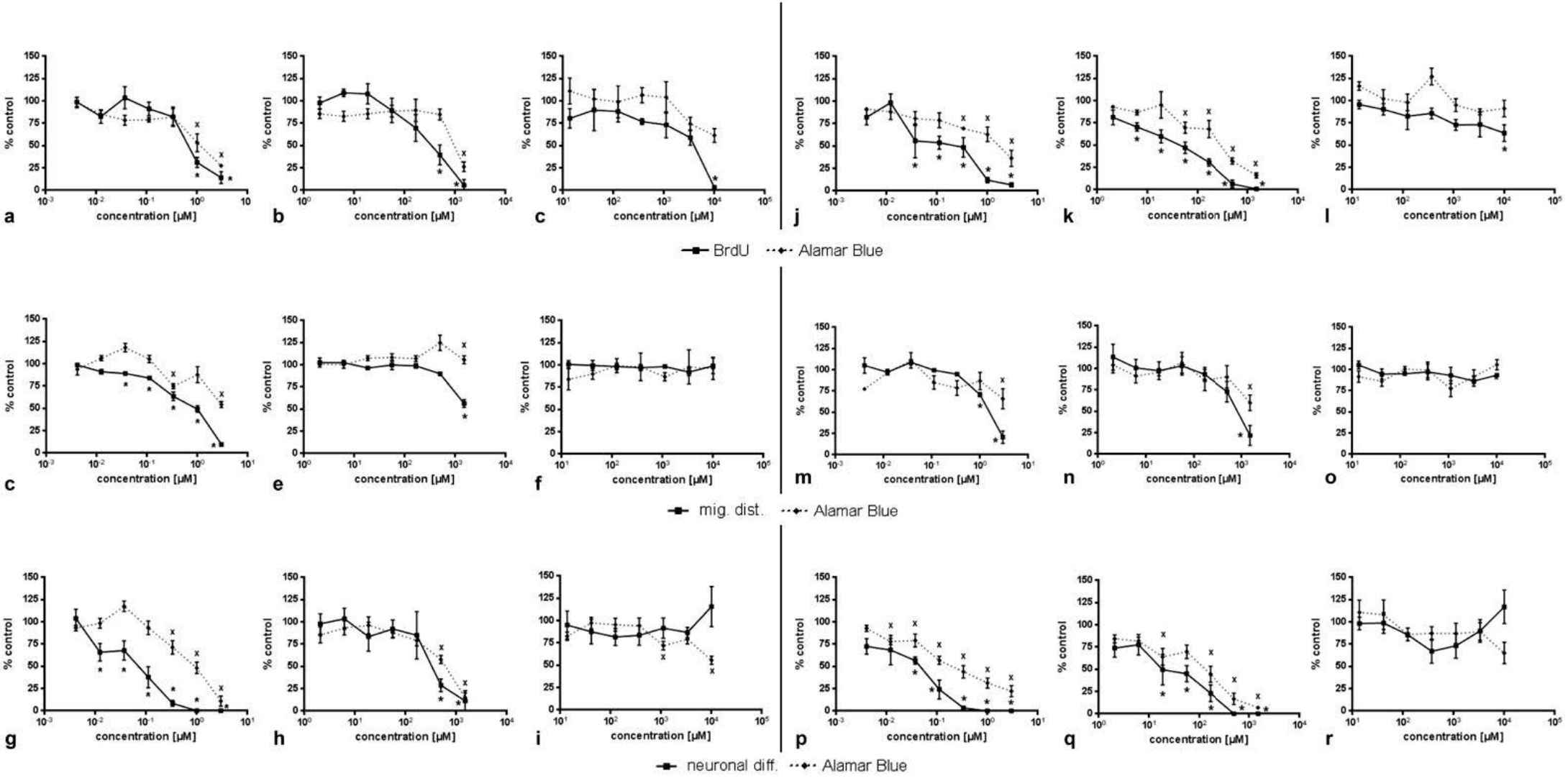

Figure 3

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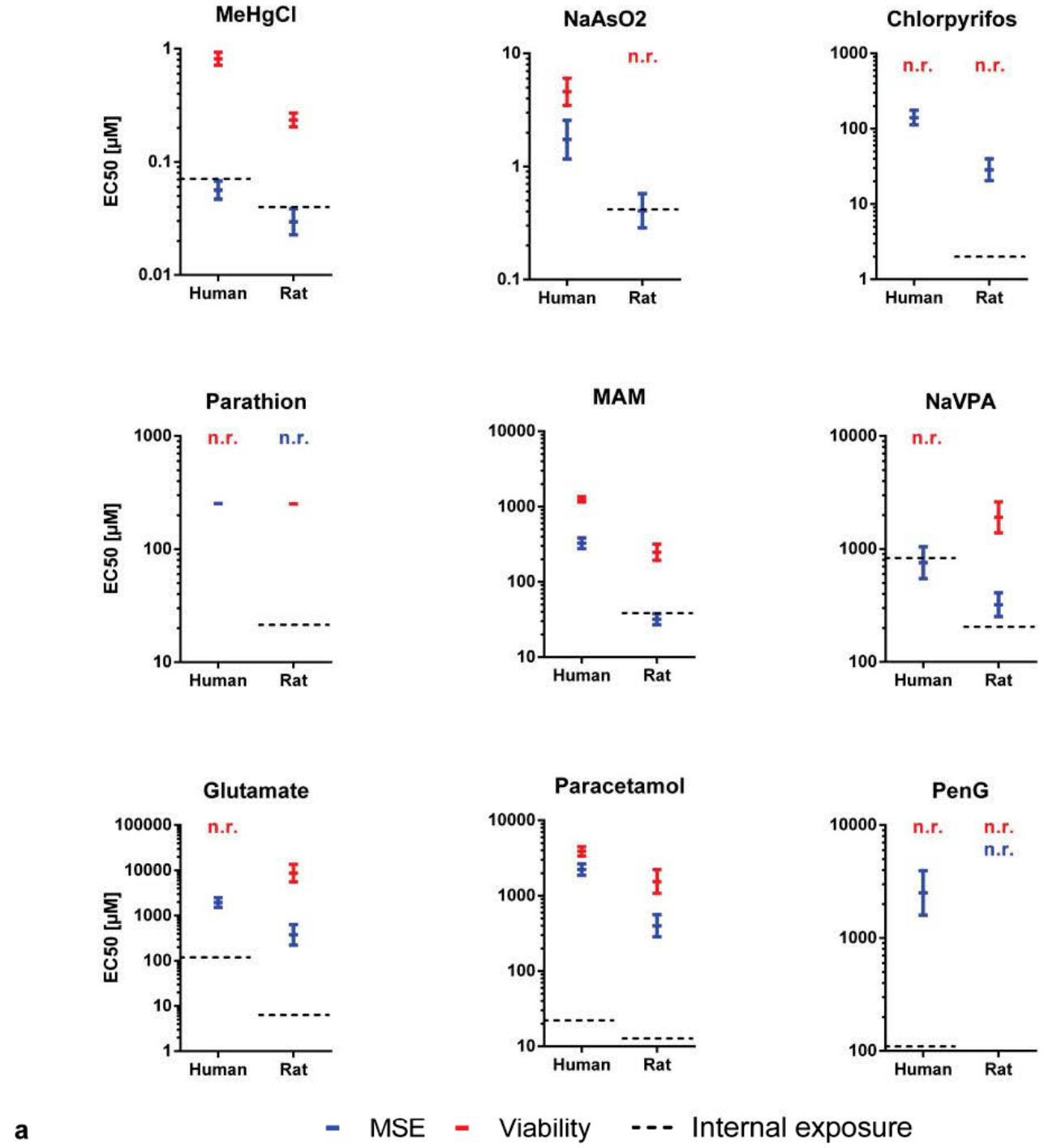

Figure 4
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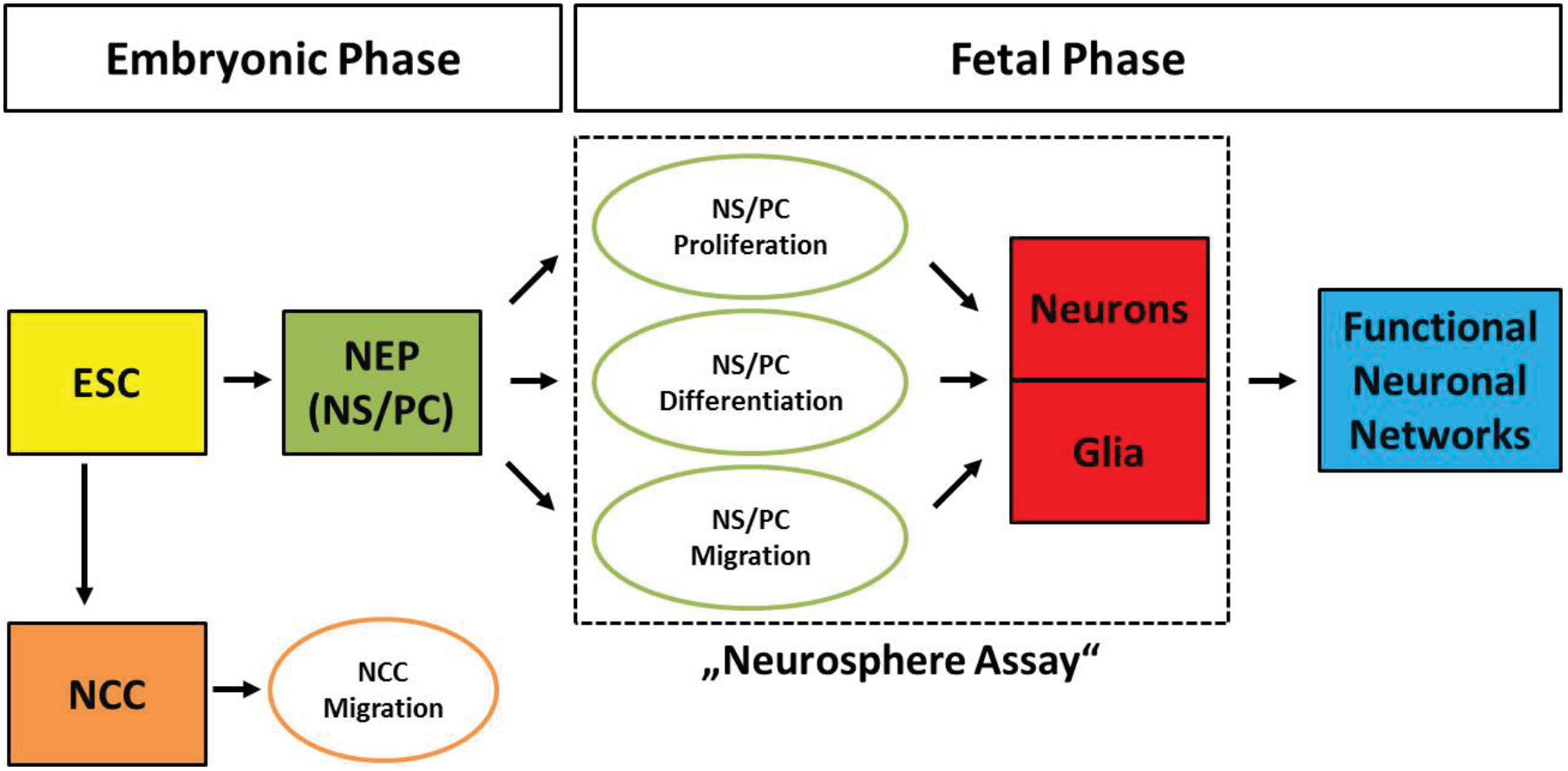

Electronic supplementary material

Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events

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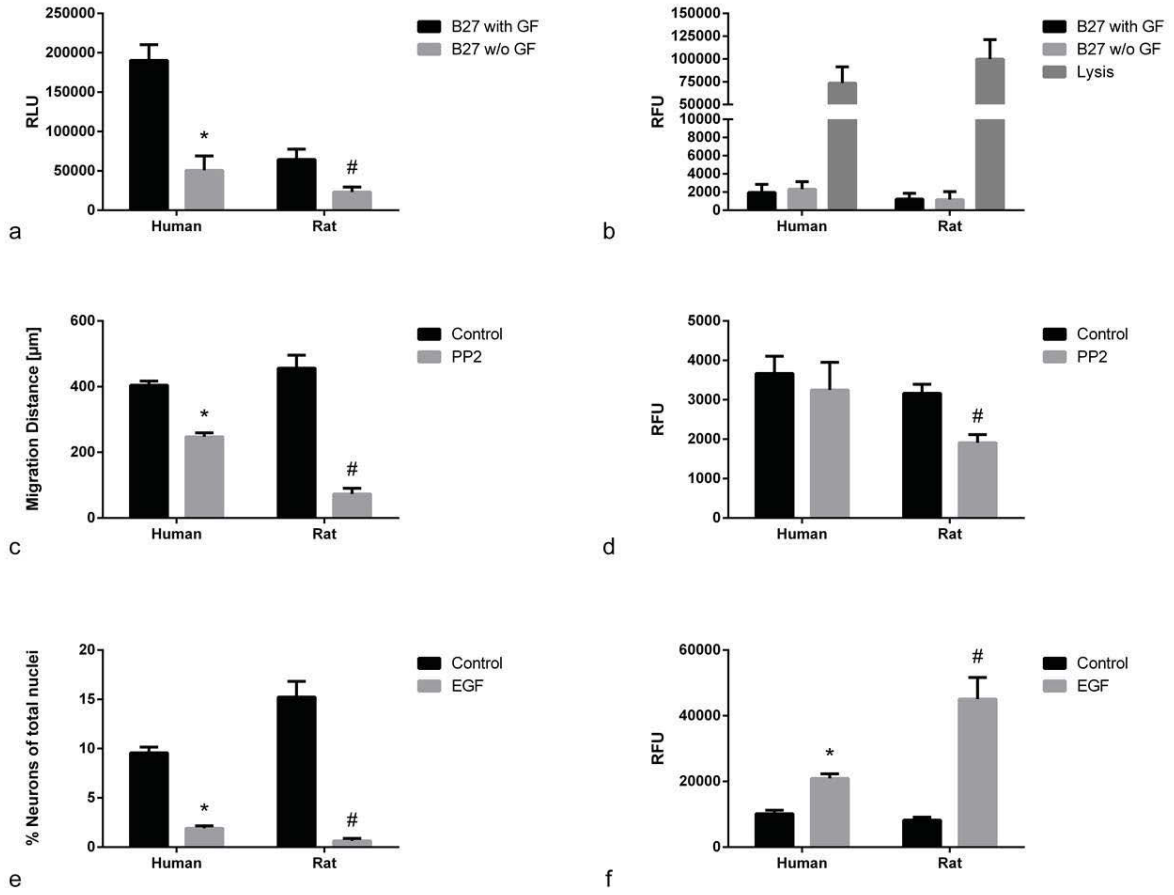
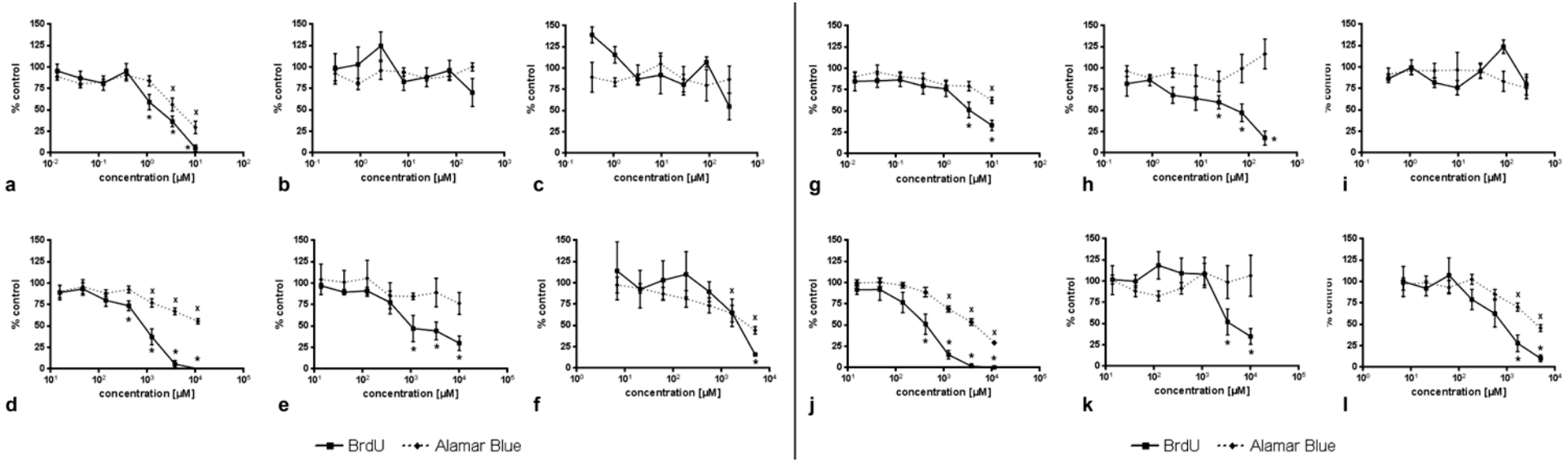
This file contains Supplementary Figures for human and rat endpoint specific controls (Fig. S1), concentration response curves for the endpoints proliferation (Fig. S2), migration (Fig. S3) and neuronal differentiation (Fig. S4) of the chemicals $NaAsO_2$, Chlorpyrifos, Parathion, Sodium Valproate, Monosodium Glutamate and Paracetamol. Moreover, a parallelogram approach which was applied to the data of sodium valproate is shown in Fig. S5. Supplementary Table 1 lists all chemical compounds of the training set used in this study. In addition, a Supplementary Materials and Methods section as well as a Supplementary Discussion is provided. References used in the Supplementary Discussion are listed at the end of this document as Supplementary References.

Figure S1:



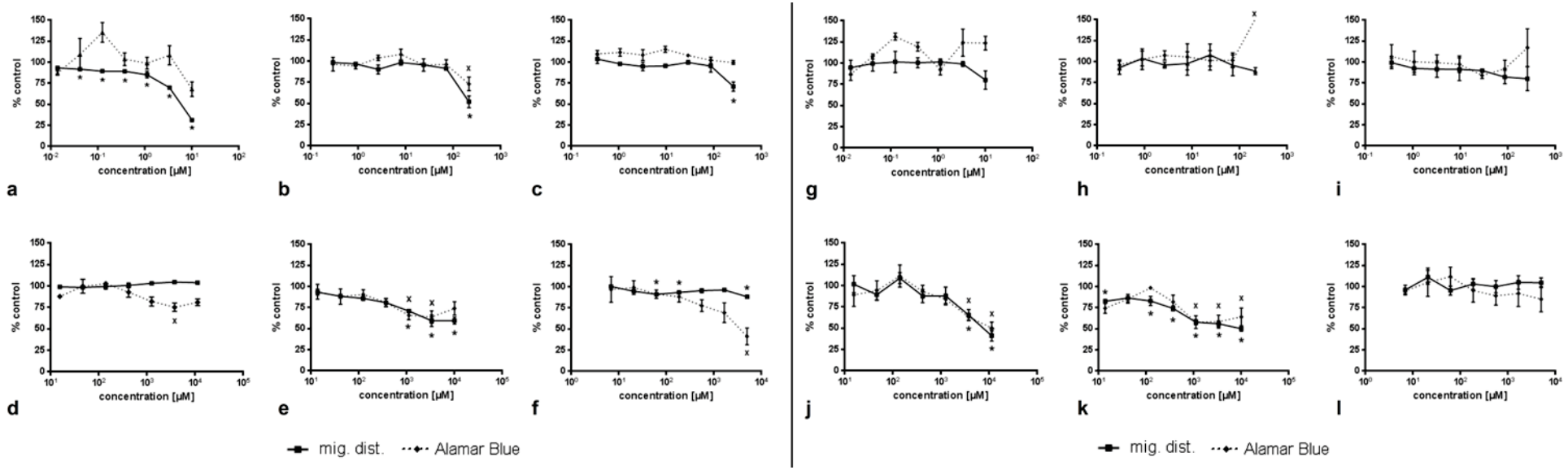
Supplementary Fig. 1 Control of general assay performance in the "Neurosphere Assay" with endpoint-specific controls. **a. and b.** Four human or rat neurospheres were plated with one floating sphere per 96-well in presence (B27 with GF) or absence of growth factors (B27 w/o GF). After 3 days **a.** proliferation was assessed by BrdU Assay and **b.** cytotoxicity was assessed by LDH Assay. Complete cell lysis by Triton X was used as a cytotoxicity control. Values are given in average of relative luminescence (RLU) or fluorescence (RFU) values ± SEM (n = 4 independent experiments). **c. and d.** Five human or rat neurospheres were plated in a PDL/Laminin coated chamber of an 8-well chamber slide in control media or in presence of 10 µM PP2. After 24 hrs phase contrast pictures of migrated spheres were taken. **c.** Migration distance was measured and **d.** viability was assessed by Alamar Blue Assay. Values are given in average migration distances in µm (**c.**) or RFU (**d.**) values ± SEM (n = 4 independent experiments), respectively. **e. and f.** Five human or rat neurospheres were plated in a PDL/Laminin coated 8-well chamber in control media or in presence of 20 ng/mL EGF. After 3 days **e.** cells were fixed, immunocytochemically stained for βIII-tubulin and counterstained with Hoechst and **f.** viability was assessed by Alamar Blue Assay. Immunofluorescent pictures were taken and neurons were quantified by manual counting. Values are given in percentages of neurons to total nuclei (**e.**) or RFU (**f.**) values ± SEM (n = 4 independent experiments). Asterisks and hashs denote significance in respect to controls in hNPCs and rNPCs, respectively ($p < 0.05$)

Fig. S2:



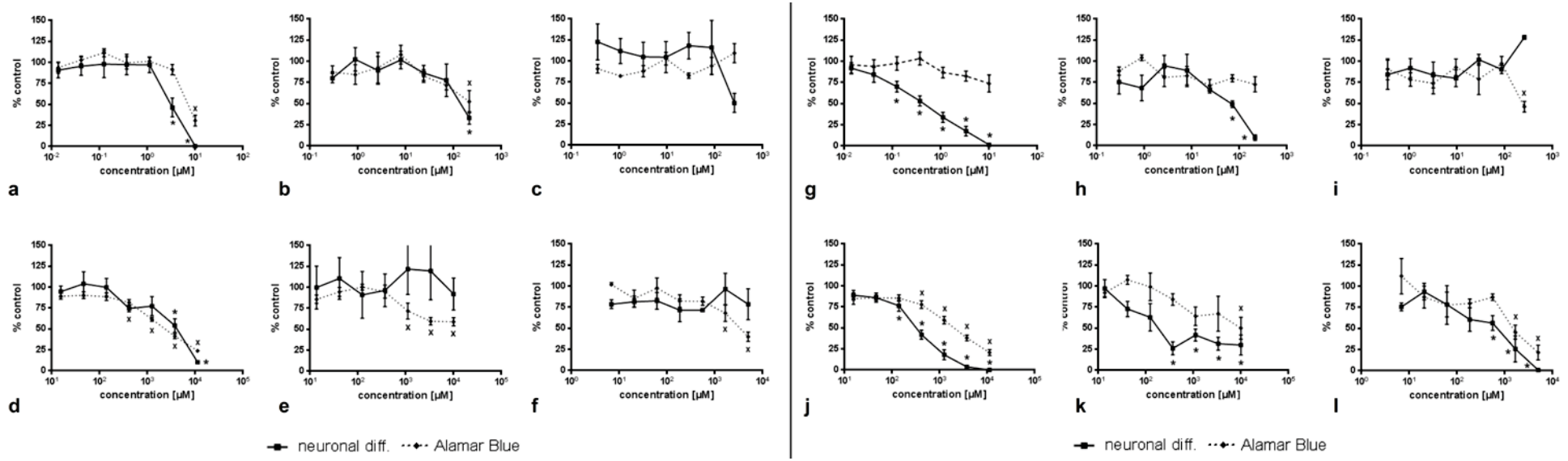
Supplementary Fig. 2 Proliferation assay. Human (**a-f**) and rat neurospheres (**g-l**) were exposed to six testing compounds (**a and g**: $NaAsO_2$; **b and h**: CPF; **c and i**: parathion; **d and j**: NaVPA, **e and k**: glutamate, **f und l**: paracetamol) with 7 concentrations per compound. Therefore, 4 floating spheres per exposure group were plated one sphere per 96-well for 3 days. At the end of exposure time, proliferation and viability were assessed by the BrdU and the Alamar Blue Assay, respectively. Concentration response curves for each testing compound are shown for human and rat neurospheres. Values are given as average percentages of solvent control for the endpoints proliferation (BrdU) and viability (Alamar Blue) ± SEM (n = 3-13 independent experiments). Asterisks denote significance respect to solvent control for the endpoint proliferation and crosses denote significance respect to solvent control for the endpoint viability ($p < 0.05$)

Fig. S3:



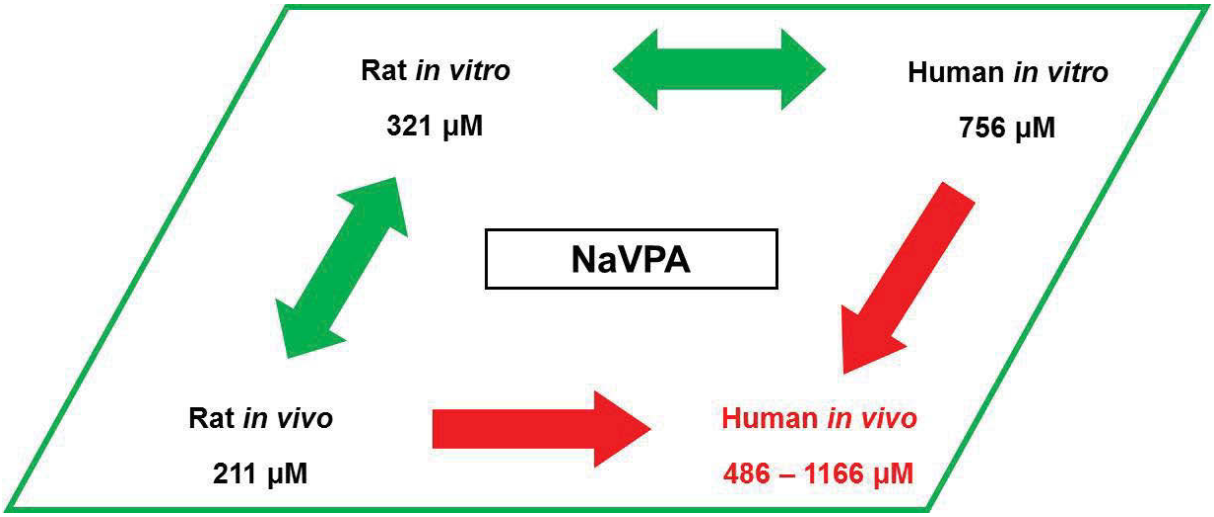
Supplementary Fig. 3 Migration assay. Human (**a-f**) and rat neurospheres (**g-l**) were exposed to six testing compounds (**a and g**: $NaAsO_2$; **b and h**: CPF; **c and i**: parathion; **d and j**: NaVPA, **e and k**: glutamate, **f und l**: paracetamol) with 7 concentrations per compound. Therefore, 5 spheres per exposure group were plated in one well of a PDL/laminin coated 8-Well chamber slide 96-well for 24 hrs. At the end of exposure time, phase contrast pictures were taken for measuring migration distances and viability was monitored by the Alamar Blue Assay. Concentration response curves for each testing compound are shown for human and rat neurospheres. Values are given as average percentages of solvent control for the endpoints migration (mig. dist.) and viability (Alamar Blue) ± SEM (n = 3 independent experiments). Asterisks denote significance respect to solvent control for the endpoint migration and crosses denote significance respect to solvent control for the endpoint viability ($p < 0.05$)

Fig. S4:



Supplementary Fig. 4 Neuronal differentiation assay. Human (**a-f**) and rat neurospheres (**g-l**) were exposed to six testing compounds (**a and g**: $NaAsO_2$; **b and h**: CPF; **c and i**: parathion; **d and j**: NaVPA, **e and k**: glutamate, **f und l**: paracetamol) with 7 concentrations per compound. Therefore, 5 spheres per exposure group were plated in one well of a PDL/laminin coated 8-Well chamber slide for 3 days. At the end of exposure time, viability was monitored by the Alamar Blue Assay and neurospheres were fixed, immunocytochemically stained for βIII-tubulin and counterstained with Hoechst. Immunoflourescent pictures were taken and neurons were quantified by manual counting. Concentration response curves for each testing compound are shown for human and rat neurospheres. Values are given as average percentages of solvent control for the endpoints neuronal differentiation (neuronal diff.) and viability (Alamar Blue) ± SEM (n = 3-15 independent experiments). Asterisks denote significance respect to solvent control for the endpoint neuronal differentiation and crosses denote significance respect to solvent control for the endpoint viability ($p < 0.05$)

Fig. S5:



Supplementary Figure 5: Parallelogram Approach for NaVPA. EC_{50} values for the most sensitive endpoints (MSEs) of NaVPA in human and rat neurospheres are applied in a parallelogram approach. Therefore, existing rat *in vivo* data are compared to rat *in vitro* data to illustrate *in vivo* - *in vitro* similarities/differences. Rat *in vitro* data are compared with human *in vitro* data to obtain information regarding interspecies differences. All these data will then allow an extrapolation of possible effects in humans *in vivo*. Green = experimental data, red = extrapolation

Supplementary Tables

Supplementary Table 1: Testing compound set used in this study. The set of chemicals comprised of six compounds with known developmental neurotoxic potential (positive substances) and three negative compounds without proven developmental neurotoxic potential (negative substances).

compound	CAS-No.	vehicle	source	stock concentration	concentration range tested
positive substances:					
methylmercurychloride (MeHgCl)	**115-09-3**	**DMSO**	**Sigma Aldrich**	**2 mM**	**3 µM-0.004 µM**
sodium (meta)arsenite ($NaAsO_2$)	**7784-46-5**	**DMSO**	**Sigma Aldrich**	**10 mM**	**10 µM-0.014 µM**
methylazoxy methanol acetate (MAM)	**592-62-1**	**H2O**	**NCI**	**1500 mM**	**1500 µM-2.058 µM**
valproic acid sodium salt (NaVPA)	**1069-66-5**	**H2O**	**Sigma Aldrich**	**1125 mM**	**11250 µM-15.432 µM**
chlorpyrifos ethyl (CPF)	**39475-55-3**	**DMSO**	**LGC Standards**	**285.2 mM**	**213.9 µM-0.293 µM**
parathion ethyl (parathion)	**56-38-2**	**DMSO**	**LGC Standards**	**343.3 mM**	**257.5 µM-0.353 µM**
negative substances:					
L(+)-Monosodium glutamate monohydrate (glutamate)	**6106-04-3**	**H2O**	**Sigma Aldrich**	**1000 mM**	**10000 µM-13.717 µM**
4-Acetamidophenol (paracetamol)	**103-90-2**	**DMSO**	**Sigma Aldrich**	**5000 mM**	**5000 µM-6.859 µM**
Penicillin G sodium salt (PenG)	**69-57-8**	**PBS**	**Sigma Aldrich**	**1000 mM**	**1000 µM-13.717 µM**

Supplementary Material and Methods

Neurosphere culture. Rat neurospheres were prepared as described previously (Baumann et al. 2014). Briefly, brains of wild-type Wistar rats (Charles River, Sulzfeld, Germany) were removed at postnatal day (PND) 5, dissected and digested in Papain/DNase solution (Worthington Biochemical Corporation, Troisdorf, Germany). Afterwards, the tissue suspension was triturated to obtain a single-cell suspension and ovomucoid solution was added to stop the tissue digestion. The cell suspension was centrifuged, pellets were resuspended and plated in 10-cm petri-dishes. The animals were treated humanely and with regard for alleviation of suffering.
Both human and rat NPCs were cultured in proliferation medium [DMEM and Hams F12 (3:1) supplemented with B27 (Life Technologies, Darmstadt, Germany), 20 ng/mL epidermal growth factor (EGF; Life Technologies), 100 U/mL penicillin, and 100 µg/mL streptomycin (PAN Biotech, Aidenbach, Germany)] in a humidified 95% air/5% CO_2 incubator at 37° C in suspension culture.
Differentiation was initiated by growth factor withdrawal in differentiation medium [DMEM and Hams F12 (3:1) supplemented with N2 (Life Technologies), 100 U/mL penicillin, and 100 µg/mL streptomycin] and plating onto poly-D-lysine (PDL)/laminin–coated chamber slides.

Cell viability assay. In every experiment cell viability was assessed in the same wells used for the more specific DNT endpoints like progenitor cell proliferation and differentiation as previously described (Baumann et al. 2014). Therefore we measured cell viability using an Alamar Blue assay (CellTiter-Blue assay Promega, Mannheim, Germany). Cells completely lysed by 0.36% Triton X-100 (Sigma Aldrich) serve as endpoint specific control. As background control wells with the respective medium but without cells were used.

Cytotoxicity assay. For the cytotoxicity measurement the lactate dehydrogenase (LDH) assay (CytoTox-One; Promega, Mannheim, Germany) was used as described previously (Baumann et al. 2014). Cells completely lysed by 0.36% Triton X-100 (Sigma Aldrich) serve as endpoint specific control. As background control wells with the respective medium but without cells were used.

Chemicals. Chemicals were of the highest purity available and dissolved in either 100% DMSO (Carl Roth GmbH, Karlsruhe, Germany; maximum solvent concentration 0.1%), sterile distilled H_2O or sterile PBS (Life Technologies, Darmstadt, Germany; maximum solvent concentration 1%) based on the solubility of each chemical and stock solutions of 1.125 M – 2 mM were prepared.

Supplementary Discussion

Arsenic is an environmental toxicant with known DNT potential as pre- or postnatal exposure through contaminated water or dried milk is associated with mental retardation in humans (Grandjean and Landrigan 2006). One of its most prominent modes of action is the generation of reactive oxygen species in the brain (Flora 2011). In hNPCs, proliferation was the most affected endpoint with an EC_{50} value of 1.7 µM. Epidemiological findings showed that arsenic concentrations of 50 µg/L in drinking water, which might result in a blood concentration of 2.25 µg/L (Concha et al. 1998), already decreased intellectual abilities in children (Wasserman et al. 2004). However, arsenic brain concentrations in humans are not known. In rNPCs arsenic inhibited neuronal differentiation with an EC_{50} value of 0.4 µM and was thus the most sensitive endpoint (MSE). An administration of 36,7 mg/L sodium arsenite in drinking water from GD 15 on for 4 months showed increased locomotor activity and learning deficits in rats. This exposure produced arsenic brain concentrations of 4,4 µg/g (35 µM) in the offspring (Rodrıguez et al. 2002). A study with much lower doses administered (0,3 mg/L in drinking water) during gestation observed altered spontaneous behavior in neonatal rats (Chattopadhyay et al. 2002) suggesting, in support of the here presented neurosphere data, adverse neurodevelopmental effects at much lower concentrations than 35 µM. Arsenic brain concentrations were not measured in these animals and exposure time differed from the Rodriguez et al. (2002) study, but may be estimated to approximately 0,3 µM (Rodrıguez et al. 2002). However, this has to be handled with caution and measured arsenic brain concentrations are needed to perform a quantitative evaluation of the sensitivity of the rat neurosphere assay. Moreover, for a comprehensive human risk assessment, an estimation of arsenic concentrations in human fetal or child brain is necessary.

For the organophosphate pesticide parathion there is evidence from rat studies that it impairs brain development at concentrations below those inhibiting acetylcholine esterase (Slotkin et al. 2006). Parathion only inhibited neuronal differentiation in hNPCs at a concentration of 253 µM. Although exposure to parathion is related to neurodevelopmental disturbances (Ruckart et al. 2004), there is – to the best of our knowledge - no data for internal exposure levels available which precludes us from a conclusive assessment of hNPC data for the actual human risk. Nevertheless, effective parathion concentrations *in vitro* seem to exceed internal exposure levels. In rNPCs parathion did not specifically inhibit any neurodevelopment-related endpoint. However, administration of 0.1 mg/kg parathion from PND 1 to 4 in rats, which might produce a brain concentration of 23 µM (Gearhart et al. 1994), resulted in learning deficits (Slotkin et al. 2009). This indicates that the rat neurosphere assay did not classify parathion as hazardous to rat neurodevelopment. The endpoints measured with the neurosphere assay are not the only key events relevant for brain development. Therefore, it is highly likely that parathion disturbs neurodevelopment via different key events than the ones assessed here. This important negative result thus supports the concept of a modulatory approach to neurodevelopmental toxicity testing taking developmental timing and a

comprehensive selection of necessary key events into consideration (Fig. 4). It also underlines the importance of knowing the biological application domain for an *in vitro* assay.

NaVPA is a prominent antiepileptic drug with proven teratogenic and DNT potential if taken during pregnancy (Ornoy 2009). In hNPCs, proliferation was inhibited at subcytotoxic concentrations (EC_{50} = 756 µM). Clinical relevant plasma concentrations lie between 50 and 120 µg/mL (347 to 833 µM; Warner et al. 1998) and accumulate to estimated fetal brain concentrations of 486 to 1166 µM (Künig et al. 1998; Ornoy 2009). Therefore, NaVPA inhibited proliferation of hNPCs within a clinically relevant concentration range. In rNPCs, both proliferation and neuronal differentiation were specifically inhibited with EC_{50} values between 300 and 400 µM. *In vivo*, a low dose of 50 mg/kg in PND 7 rats, which caused apoptotic neurodegeneration (Bittigau et al. 2002), results in an approximate brain concentration of 211 µM (Eskandari et al. 2011), which is close to the EC_{50} values found for inhibition of rNPC proliferation and differentiation. Data on VPA-induced neurogenesis *in vivo* is sparse. An elegant *in vivo* study in mice, however, supports our notion as a daily VPA dose of 250 µg/g between PND 7 and 14 reduced proliferation and neurogenesis in postnatal mouse brains (Foti et al. 2013). To the best of our knowledge, there has no behavioral study or study investigating proliferation or neurogenesis in rats been performed with equivalent low doses and an appropriate (postnatal) timing of administration. Thus, although prediction of human and rat neurospheres for DNT risk of NaVPA with the available data looks promising, one has to take into consideration that data used in this approach compares a functional endpoint (intelligence quotient) in humans with a histopathological endpoint (apoptotic neurodegeneration) in rats (Suppl. Fig. 5). It also has to be pointed out here, that effects assessed with the neurosphere assay do not relate to the VPA-induced disruption of neural tube closure. Therefore, one needs a different *in vitro* assay (Fig. 4).

The two negative compounds glutamate and paracetamol affected DNT endpoints in human or rat neurospheres, and those effects were also separated from general cytotoxicity. However, EC_{50} values were consistently higher than those of positive compounds and dramatically exceeded therapeutic internal doses as discussed below. hNPC proliferation was inhibited by the excitotoxic neurotransmitter glutamate with an EC_{50} value of 1938 µM. However, normal plasma levels of glutamate in humans lie between 40 and 70 µM and may be elevated up to 110 µM after glutamate-rich food intake (Stegink et al. 1979). Moreover, the blood brain barrier is relatively impermeable for glutamate (Hawkins 2009), showing that concentrations which inhibited proliferation in hNPCs were probably far from any human relevance. In rNPCs neuronal differentiation was inhibited with an EC_{50} value of 375 µM. *In vivo*, 4g/kg glutamate in rats from PND 1 to 10 affected learning behavior in subadult animals. However, glutamate brain concentrations did not exceed 6 µM in the animals, showing that the EC_{50} values causing decreased neuronal differentiation in rat neurospheres are of no physiological relevance. Paracetamol, which is hepatotoxic at high concentrations, inhibited proliferation in hNPCs at millimolar concentrations (2219 µM) and neuronal differentiation in rNPCs at micromolar

concentrations (399 µM). A therapeutic administration of 15 mg/kg in either humans or rats resulted in a plasma concentration of 66 µM and in CSF concentrations of 26 µM (human) and 13 µM (rat), respectively (Westerhout et al. 2012), which is again much lower than EC_{50} values determined in the neurosphere assays.

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Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events

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2.5. 21st Century Risk Assessment: case study for species-specific hazard characterization of valproic acid

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Derzeit besteht ein dringender Bedarf für die Entwicklung von Alternativmethoden, die das entwicklungsneurotoxische Potential von Chemikalien vorhersagen können, da die verwendeten *in vivo* Studien zu viele Ressourcen verbrauchen und es von großer Bedeutung ist, dass das Gehirn als wichtiger sozioökonomischer Faktor während seiner Entwicklung geschützt wird. Im Zusammenhang mit dem Paradigmenwechsel, den die toxikologische Testung Anfang des 21sten Jahrhunderts durchlaufen hat, verwenden wir in der vorliegenden Studie humanen und Rattenneurosphären als Teil einer alternativen Testbatterie, um das inhärente Gefahrenpotential von Substanzen bezüglich ihrer Entwicklungsneurotoxizität zu untersuchen. Wir präsentieren eine Fallstudie des bekanntermaßen für den Menschen entwicklungsneurotoxischen Antiepileptikums Natriumvalproat und zeigen, dass Natriumvalproat mit Prozessen, die für die Gehirnentwicklung relevant sind, spezies-spezifisch interferiert. Diese Fallstudie liefert einen Machbarkeitsbeweis für die Verwendung humaner und Rattenneurosphären als Teil der Bemühung für Entwicklungsneurotoxizitäts-Screening, um molekulare Veränderungen in Signalwegen und zellulären Funktionen spezies-spezifisch untersuchen zu können. Damit eignet sich der Neurosphärenassay als Teil einer größeren Batterie an Methoden für das Screenen auf Schlüsselereignisse der Gehirnentwicklung für eine Anwendung in einem verbesserten Ansatz der Entwicklungsneurotoxizitätsrisikobewertung.

21st Century Risk Assessment: case study for species-specific hazard characterization of valproic acid

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Abstract

There is an urgent need for alternative methods predicting developmental neurotoxicity (DNT) of chemicals because current *in vivo* tests are too resource-intensive and the brain as an important socioeconomic factor needs protection during its developmental process. With regards to the paradigm shift of toxicological testing in the 21st century, we propose neurospheres as part of an alternative testing battery to assess compound hazards for neurodevelopmental toxicity. We present a case study of the human DNT-pharmaceutical valproic acid (VPA), showing that VPA interferes with pathways that possess relevance for neurodevelopment in a species-specific manner. This case study provides a proof-of-principle for using primary human and rat neurospheres as part of a DNT screening effort to predict alterations of signaling pathways and cellular functions in a species-specific manner. Thus, the comparative neurosphere assay as part of a larger key event screening battery could be applied for improving human DNT risk assessment.

Keywords

Hazard assessment, species differences, neural progenitor cells, compound fingerprint, human, rat

Introduction

In 2007 the National Research Council of the USA proposed a paradigm shift in drug and chemical testing from apical endpoint evaluation in animals towards mechanism-based *in vitro* analyses combined with *in silico* approaches (Collins et al., 2008). This proposed revolution in toxicological testing executed by the Tox21 initiative is based on the large amount of clinical drug failures due to adverse drug responses (Arrowsmith, 2011) and the numerous environmental chemicals devoid of toxicity data for safety evaluation and regulation (Judson et al., 2009). Such shortcomings of human-relevant compound data in pharmacology and toxicology are based on the resource-intensiveness of animal experiments (time, money), the ethical concerns of society and the overall insufficient translation of scientific findings from animals to humans (Council, 2007). Lately, the latter has received increased scientific attention (van der Worp et al., 2010) as e.g. translation of drug candidates from highly cited animal research into clinical application was reported to be poor (Hackam and Redelmeier, 2006). Experimental examples for species-specific differences emerged among others in the rate of drug absorption (Tanaka et al., 2013), inflammatory diseases (Seok et al., 2013) and neurological disorders including ischemic stroke (van der Worp and van Gijn, 2007, O'Collins et al., 2006). These experiences in pharmacology are supported by cross-species molecular studies demonstrating species-specificity in signaling pathways (e.g. Black et al., 2012, Thomas et al., 2013, Gassmann et al., 2010) and lead to the international consensus that toxicological *in vitro* testing should move to human-based methods to minimize species effects in responses to compound exposure (Seidle and Stephens, 2009, Krewski et al., 2010).

The new concept of toxicological testing for application in the regulatory context includes hazard assessment applying computational analyses of bioactivity profiles of chemical libraries. Such bioactivity profiles can be obtained by measuring e.g. protein biomarkers of human-relevant biological pathways in *in vitro* tests thus receiving specific, pathway-related compound ‘fingerprints’ (Kleinstreuer et al., 2014, Sturla et al., 2014). When such cell type-/tissue-specific fingerprints are related to *in vivo* outcomes, for example within the adverse outcome pathway (AOP) concept, they might be suited for chemical screening and prioritization and have applications to toxicity testing and drug discovery (Knudsen et al., 2015, Villeneuve et al., 2014).

The most critical issue in such an approach is the choice of *in vitro* method for pathway analyses, as cells have to maintain signaling properties of their tissue of origin. Compared to two-dimensional cultures, this is drastically improved by cultivation of cells in a three-dimensional (3D) format (Alepee et al., 2014, Yamada and Cukierman, 2007). The uncertainty in functional implication of compound-related pathway perturbation in a species-specific context poses a second challenge. To address these issues, 3D cultures with the ability to mimic tissue- and species-specific cellular functions are of advantage.

Here, we present a case study on species-specific, pathway-related, functional endpoint-associated compound fingerprints in a 3D neurosphere *in vitro* model, which mimics neurodevelopmental processes in a dish (Fig. 1; Baumann et al., 2014, Gassmann et al., 2010, Moors et al., 2007, Moors et al., 2009). For this case study we use the antiepileptic drug valproic acid (VPA; rev. in Ornoy, 2009) as a known human developmentally neurotoxic and relatively data-rich compound. Children prenatally exposed to this drug may develop autistic spectrum disorders, developmental delays and behavioral changes (Ornoy, 2009). While cell biological effects underlying human developmental neurotoxicity (DNT) of VPA are not known (Ornoy, 2009), VPA affects neurodevelopmental processes like neural progenitor cell (NPC) proliferation and differentiation in rodents in a developmental stage-specific manner (Foti et al., 2013, Go et al., 2012). Relating rodent timing of brain development to human developmental phases (Fig. 1; Clancy et al., 2007a, Clancy et al., 2007b), one can allocate the VPA-dependent increase of NPC proliferation and neuronal differentiation to the human embryonic stage, where organ formation takes place, while opposite effects of VPA on these processes can be assigned to the late fetal stage (3rd trimester of gestation) of human development. Our results show that therapeutic drug concentrations of VPA reduce the functional neurodevelopmental key events proliferation and neurogenesis in human and rat primary NPC from GW 18 (human early fetal stage, 2nd trimester of gestation) and postnatal day (PND) 5, respectively. However, functional fingerprints differ between human and rodent neurospheres. We elucidated the VPA-triggered quantitative aspects of molecular pathways causing species-specific functional fingerprints in 3D neurospheres generated from mid-term neurodevelopmental tissue. Molecular dissimilarities in expression of signaling pathway components *in vitro* were verified for *in vivo* relevance. This proof-of-concept study suggests that data generated in 3D organoid cultures might be useful for AOP-building by bridging the gap between rodents and humans as well as between molecules in cellular functions. In addition, an assembly of such pathway-related functional fingerprints could feed algorithms for combined *in vitro/in silico* hazard assessment (Knudsen et al., 2015) that are currently developed and thereby contribute to DNT risk assessment of pharmacologically or toxicologically relevant compounds within the framework of 21st century toxicity testing.

Material and Methods

***Cell culture*.** Normal human neural progenitor cells (hNPC) used in this study were purchased from Lonza (Verviers, Belgium).

Rat and mouse neurospheres were prepared as described previously (Gassmann et al., 2012, Baumann et al., 2014). Briefly, brains of wild-type Wistar rats or C57/BL6 mice (Charles River, Sulzfeld, Germany) were removed at PND 5 and 1, respectively, dissected

and digested in Papain/DNase solution (Worthington Biochemical Corporation, Troisdorf, Germany). Afterwards, the tissue suspension was triturated to obtain a single-cell suspension and ovomucoid solution was added to stop the tissue digestion. The cell suspension was centrifuged, pellets were resuspended and plated in 10-cm petri-dishes. The animals were treated humanely and with regard for alleviation of suffering.

Both human and rodent NPC were cultured in proliferation medium [DMEM and Hams F12 (3:1) supplemented with B27 (Life Technologies, Darmstadt, Germany), 20 ng/ml epidermal growth factor (EGF; Life Technologies), 20 ng/ml fibroblast growth factor (FGF, only in human and mouse neurosphere cultures; R&D Systems, Wiesbaden-Nordenstedt, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (PAN Biotech, Aidenbach, Germany)] in a humidified 95% air/5% CO_2 incubator at 37°C in suspension culture. These conditions are referred to as “proliferative conditions” in the following.

Differentiation was initiated by growth factor withdrawal in differentiation medium [DMEM and Hams F12 (3:1) supplemented with N2 (Life Technologies), 100 U/ml penicillin and 100 µg/ml streptomycin] and plating onto poly-D-lysine (PDL)/laminin–coated surfaces. These conditions are referred to as “differentiating conditions” in the following.

Cell viability assay. Cell viability was assessed in the same wells used for the more specific DNT endpoints proliferation and neuronal differentiation. Therefore we measured cell viability using an Alamar Blue assay (CellTiter-Blue assay Promega, Mannheim, Germany) as previously described (Baumann et al., 2014). Cells completely lysed by 0.36% Triton X-100 (Sigma Aldrich) serve as endpoint specific control. As background control wells with the respective medium but without cells were used.

Proliferation analysis. For quantification of progenitor cell proliferation the Cell Proliferation ELISA, BrdU (chemiluminescent) from Roche (Mannheim, Germany) was used according to the manufacturer’s instructions with a BrdU incubation period of 16 hrs as described previously (Baumann et al., 2014). Spheres cultivated in proliferation medium without growth factors served as endpoint specific control and for correction of unspecific binding of the BrdU antibody some spheres were cultured without BrdU.

Migration analysis. Migration analyses were performed as previously described (Baumann et al., 2014). 10 µM PP2 (Sigma Aldrich, Taufkirchen, Germany), a selective inhibitor for Src-family kinases, was used as endpoint specific control (Moors et al., 2007).

Neuronal differentiation analysis. After assessment of cell viability differentiated spheres were fixed in 4% paraformaldehyde for 30 min at 37°C. Neurons were identified by immuncytochemical staining against β(III)-tubulin as previously described (Baumann et al.,

2014). For quantification analyses, we used an ImageJ macro to count the Hoechst 33258 positive nuclei automatically. Neurons were counted manually using ImageJ. As endpoint specific control spheres were cultured in differentiation medium with 20 ng/ml EGF because EGF inhibits the neuronal differentiation (Ayuso-Sacido *et al.* 2010).

Apoptosis analysis. In differentiated neurospheres, apoptotic cells were identified by Life-Dead-Staining with Hoechst 33258 and propidium iodide as described previously (Baumann et al., 2014). Briefly, 30 min prior the end of exposure time Hoechst 33258 and propidium iodide were added and spheres were fixed in 4% paraformaldehyde afterwards. Apoptotic cells were identified in fluorescent pictures. For quantification analyses, we used an ImageJ macro to count the Hoechst 33258 positive nuclei automatically. PI/Hoechst double positive cells were counted manually using ImageJ.

Chemical exposure for assessment of neurodevelopmental endpoints. Dimethyl sulfoxide (DMSO), the vehicle for the majority of chemicals in this study, was purchased from Carl Roth GmbH (Karlsruhe, Germany). Unless stated otherwise, chemicals were purchased from Sigma Aldrich (Munich, Germany). Final DMSO concentrations did not exceed 0.25%. Sodium valproate and sodium butyrate were dissolved in sterile distilled water; valpromide, trolox and anacardic acid were dissolved in DMSO and Z-DEVD-fmk (DEVD; R&D Systems) was dissolved in DMSO and prediluted 1:10 in PBS (Life Technologies) with 1% BSA (Serva, Heidelberg). For concentration response experiments, the solvent concentration was equal for every dilution step.
Under proliferative conditions human and rat neurospheres were plated one sphere per well into 96-well plates in 100 µl of the exposure media. Four wells per exposure condition were used to assess proliferation by BrdU assay and viability by Alamar Blue assay. To measure neuronal differentiation, migration or apoptosis five human or rat neurospheres were plated in one well of a poly-D-lysin/laminin coated 8-chamber-slide in 500 µL of the exposure media under differentiating conditions. The cell viability was again assessed by Alamar Blue assay. For proliferation, migration and neuronal differentiation cells were exposed to chemicals for 72 hrs and for apoptosis analysis cells were exposed for 48 hrs.
For co-exposure experiments, spheres were pre-incubated with trolox or DEVD for 2 hrs, or with anacardic acid for 1 h. Afterwards, VPA was added.

Chemical exposure for gene expression analysis. For experiments under proliferative conditions, 10 spheres per well were plated into poly-D-lysin/laminin coated 24-well plates in 1 ml of the exposure medium with three wells per exposure condition for 12 hrs. For experiments under differentiating conditions either 10 spheres per well (for ANT1 analysis) or 100 pieces of 0.1 mm chopped spheres (for Ascl1 analysis) were plated into 24-well plates in

1 ml of the exposure media and differentiated 72 hrs. At the end of exposure time, spheres were collected for total RNA isolation.

Transfection of neurospheres with ANT1 siRNA. Rat neurospheres were transfected with ANT1 siRNA or scrambled siRNA (SR514511, Amsbio, Frankfurt, Germany) by Nucleofection with the 4D-Nucleofector® X unit (Amaxa, Lonza, Cologne, Germany) as described previously (Baumann et al., 2015a). Briefly, rat neurospheres with 0.3 mm in diameter were chopped to 0.1 mm, suspended in P3 solution (Nucleofector® X solutions P3 for primary cells, Lonza) and processed according to the manufacturer's instructions. 16 hrs after transfection, spheres were plated with 8 spheres per well of a poly-D-lysin/laminin coated 8-chamber-slide in 400 µl of the exposure media under differentiating conditions for analyzing neuronal differentiation after 72 hrs. For control of transfection efficiency, approximately 50 spheres were plated into poly-D-lysin/laminin coated 24-well plates in 1 ml medium with three wells per exposure condition for 72 hrs. At the end of exposure time, spheres were collected for total RNA isolation.

RNA isolation, cDNA sysnthesis and real time RT-PCR. Total RNA of proliferating or differentiated spheres was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For preparation of total rat brain RNA, rat brains were shredded in a tissue disruptor in advance. The maximal volume of sphere RNA and 500 ng human (Human, GW 22, Biocat, Heidelberg, Germany) or rat PND5 brain RNA was transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Real-time reverse-transcriptase polymerase chain reaction was performed using the Rotor-Gene Q instrumentation (Qiagen).

The PCR mix consisted of 1/2 volume of Quanti Tect SYBR Green FAST PCR Master Mix (Qiagen), 0.67 µM solutions of each primer and 2.5 µl of cDNA (1:2.5 diluted) in a final volume of 15 µl. The application started with an initial incubation step of 7 min at 95°C to activate the DNA polymerase. The conditions for PCR amplifications were 47 cycles of 10 sec at 95°C for denaturation, 35 sec at 60 °C for primer annealing, elongation and fluorescence detection. Primer sequences for Ascl1 (Hash1/Mash1), $p21^{Cip/WAF1}$, HDAC1, ANT1 and β-Actin for human and rat are given in Suppl. Table 1. Expression levels were normalized to the expression of β-actin. We evaluated gene expression using the cycle threshold (Ct) value from each sample. Calculations are based on the ΔΔCt method (Livak and Schmittgen, 2001). For determining absolute copy numbers, we used product-specific standards amplified from cDNA to generate standard curves.

Valproate metabolite analysis. For metabolite analysis human and rat neurospheres were cultured in presence of 1000 µM VPA or the respective solvent concentration under

proliferative and differentiating conditions for 48 hrs. Therefore, either 12 spheres with one sphere per well were plated in 96-well plated in 100 µl exposure media per well under proliferative conditions, or 15 spheres with 5 spheres per well of a poly-D-lysin/laminin coated 8-chamber-slide were plated in 500 µl exposure media per well under differentiating conditions. At the end of exposure time, media of the different wells per exposure group were pooled. Concentrations of the oxidative metabolites of VPA in neurosphere culture supernatants were quantified using a gas chromatography – mass spectrometry (GC-MS) assay. GC-MS instrumentation was performed as described previously (Tong et al., 2003). Briefly, 50 µl of an internal standard solution containing 12 µg/ml of [2H6]-VPA and 6.5 µg/ml of [2H7]-5-OH-VPA was added to the neurosphere culture supernatant samples (0.8 or 1 ml) in borosilicate glass screw-neck culture tubes and the mixture was acidified by adding 1 ml of 1 M KH_2PO_4 buffer (pH 3.5). The samples were extracted with ethyl acetate (8 ml) by vortex mixing for 5 min and then centrifuged at 2060 × g for 10 min at ambient temperature. The organic extract was transferred to a new borosilicate glass screw-neck culture tube containing anhydrous sodium sulfate, vortex mixed for 1 min, and centrifuged at 2060 × g for 10 min at ambient temperature. The organic layer was transferred to another new borosilicate glass screw-neck tube and evaporated to 100 – 150 µl volume in a Zymark® TurboVap LV® evaporator (Zymark Co., Hopkington, MA, USA). Subsequently, 30 µl of N,N-diisopropylethylamine and 10 µl of pentafluorobenzyl bromide were added to the sample, vortex mixed, and heated to 45°C for 1 h for the pentafluorobenzyl ester derivatization of the carboxylic acid groups. The samples were allowed to cool down to ambient temperature. Subsequently, 20 µL of dimethyl formamide and 40 µl of N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide containing 2% tert-butyldimethylsilyl chloride were added, vortex mixed, and heated to 60 °C for 2 hrs for the tert-butyldimethylsilyl derivatization of the hydroxyl groups. The derivatized samples were then cooled down to ambient temperature and then, dried under nitrogen stream for 30 min. The dried residue was reconstituted with 200 µl of n-hexanes, vortex mixed, and centrifuged at 2060 × g for 10 min at ambient temperature. The organic layer was transferred to a crimp top glass vial containing a glass insert and injected on to the GC. The concentration range for the calibration curves was 1 to 500 ng/ml for each metabolite. [2H6]-VPA (m/z 149) served as the internal standard for (E,Z)-2,3'-diene-VPA, (E,E)-2,3'-diene-VPA, (E)-2,4-diene-VPA, 4-ene-VPA, 3-ene-VPA, (E)-2-ene-VPA, and 4-keto-VPA, whereas [2H7]-5-OH-VPA (m/z 280) was the internal standard for 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, and 3-keto-VPA.

Proteome analysis. Under proliferative conditions, 6 replicates of 5 human, rat or mouse neurospheres with 0.3 mm in diameter were washed with ice cold PBS, completely dried and stored at -80 °C. Under differentiating conditions, 6 replicates of 20 human, rat or mouse neurospheres (0.3 mm, chopped to 0.1 mm for plating) were plated in one well of a poly-D-

lysin/laminin coated 24-well plate each and differentiated for 72 hrs. Afterwards, spheres were harvested and washed with ice cold PBS, completely dried and stored at -80 °C.
For the quantitative analysis of proteins expressed by human, rat and mouse neurospheres a label-free approach using signal intensities in the mass spectrometer was employed for quantification. Therefore, a RSLC nano HPLC-System in combination with an Orbitrap Elite (Thermo Fisher) and Progenesis software (Nonlinear Dynamics) was used. After tryptic digestion, peptides were separated by a 140 min gradient using a 25 cm column packed with 2 µm particles. MS analysis was done in a data dependent manner selecting the 20 most intense peaks for fragmentation. Approximately 300 ng of total protein extract per case were used for each analysis. This amount enabled the quantification of expression levels of more than 2700 proteins.

Statistics. Data analysis was performed using Graph-Pad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). In concentration response experiments, all data were normalized to the respective solvent control and are presented throughout the article as mean percent of solvent control ± standard error of the mean (SEM). Chemical effects were determined using a one-way analysis of variance (ANOVA) followed by post hoc testing using Dunnett's post hoc test. Data obtained at each chemical concentration were compared with vehicle controls. For the sigmoidal dose response curve fitting and the calculation of the EC_{50} values and 95 % confidence intervals a four-parameter logistic nonlinear regression model where the top was set at 100 % and the bottom was set at 0 % was used. Comparison of two different groups was performed using two-way ANOVA followed by Fisher's LSD test or a student's t-test. Data are presented as mean ± SEM or SD of 3 to 15 independent experiments with four to five neurospheres each. For all comparisons, results were considered significant if $p < 0.05$.

Results

On the basis of the current body of evidence that qualitative or quantitative molecular species differences in signaling pathways determine responses of cells and organs to xenobiotics, we performed proteome analyses of proliferating and differentiating human, rat and mouse neurospheres. The aim of these analyses was to determine if primary NPC from different species diverge in their quantitative and/or qualitative protein expression as a first indication for dissimilar susceptibilities towards compounds interfering with brain development. The mouse was included in the analyses as a rodent control for the rat. Human NPC proteomes were set as the gold standard and of the 2630 (proliferating neurospheres, prol) and 3266 (differentiating neurospheres, diff) proteins identified in this species, 2121 (prol) and 2346

(diff) proteins were also identified in rat proteomes, and 2055 (prol) and 2559 (diff) proteins were also identified in mouse proteomes. GO annotation analyses of these proteins revealed that species significantly differed in the protein expression. In proliferating NPC 30, 52 or 6 GO-Terms ('Biological Processes' and 'Molecular Functions') were enriched for human and rat, human and mouse or mouse and rat, respectively. In differentiating NPC 47, 37 or 23 GO-Terms were enriched for human and rat, human and mouse or mouse and rat, respectively (Suppl. Table 2). The higher number of enriched GO-Terms differing between rodents and humans than between rat and mouse indicates human-specific protein expression in developing brain cells. This is supported by principle component analyses (PCA) of these proteomes. Proliferating as well as differentiating NPC reveal substantial differences between species with mice and rat clustering closer to each other than each rodent species to human NPC (Fig. 1 B,C). Besides the qualitative differences of proteomes (equivalents of human proteins not found in rat NPC: 509 (prol), 920 (diff) and mouse NPC: 575 (prol) 707 (diff)), PCA analyses reveal quantitative differences of protein expression across species.

The neurosphere assay allows functional analyses of NPC proliferation, migration, neuronal differentiation as well as apoptosis and is thus well suited for generation of functional fingerprints within pathway-to-function analyses of compounds. The antiepileptic drug VPA reduced proliferation of human and rat NPC (EC_{50} 756 µM and 380 µM, respectively) as measured by BrdU incorporation during 3 days independently of cell death (EC_{50} for viability for human and rat cultures not reached and 4019 µM, respectively; Fig. 2 A,B). Rat NPC displayed an approximately 2-fold higher sensitivity towards VPA-induced inhibition of proliferation than human cells. The antiproliferative effect of VPA is due to histone deacetylase (HDAC) inhibition (HDACi) in both species because anacardic acid (a histone acetylase (HAT) inhibitor counteracting the HDACi effect) was able to fully restore VPA-reduced NPC proliferation in both species after 3 days at respective EC_{50} values (Fig. 2 C,D). In addition, HAT inhibition (HATi) antagonized VPA-dependent gene up-regulation of $p21^{Cip/WAF1}$ (Fig. 2 E,F), an HDAC1-dependent cell cycle regulator (rev. in Ocker and Schneider-Stock, 2007) known to mediate reduction of NPC proliferation by VPA in rodents *in vivo* (Jung et al., 2008). Next, we explored the reason for the 2-fold lower human susceptibility towards VPA-dependent inhibition of NPC proliferation. Abundance of the HDAC1 protein was 2-fold higher in rat than in human NPC (Fig. 2G) thus providing more targets for the excess amount of the small molecule VPA in rat NPC, which exerts its pharmacological effects in the mM range (Sztajnkrycer, 2002). This species-specific HDAC1 protein expression is actually reflected in the HDAC1 mRNA abundance of human and rat NPC with significantly higher expression in the latter (Fig. 2H). Human brain mRNA analyses for HDAC1 indicate the physiological *in vivo* relevance of the neurosphere results as human fetal brain expresses significantly less HDAC1 mRNA than rat brain (Fig. 2H). The relevance

of these findings is underlined by the fact that during brain development HDAC1 and 2 are not functionally redundant and thus do not compensate for each other (Foti et al., 2013).

Migration of NPC is another essential process during brain development (rev. in Andersen, 2003). Although HDACs might be involved in migration of some cell types, VPA is not specifically altering NPC migration of human or rat NPC (Supplementary Fig. 1).

During migration, NPC differentiate into neurons and glia cells. VPA treatment of differentiating NPC revealed profound species differences in inhibition of formation of young, β(III)-tubulin$^+$ neurons after 3 days of differentiation. For one, rat NPC are approximately 10-times more sensitive towards VPA-induced neuron reduction than human NPC (EC_{50} 321 µM and 3177 µM, respectively). Secondly, in contrast to rat, human neuronal loss is accompanied by VPA-induced cell death of differentiating NPC (EC_{50} value for cytotoxicity 2399 µM), while loss of young rat neurons happens independently of general cytotoxicity (EC_{50} value for cytotoxicity 2399 µM; Fig. 3A+B, D-G). Analyses of apoptosis by DNA labeling with propidium iodine (PI) revealed that after 48 hrs VPA induces apoptosis at lower concentrations in rat than in human NPC (equal effects observed at 300 µM and 3000 µM VPA, respectively; Fig. 3C, H-K). Here it has to be kept in mind that NPC differentiation areas consist of mixed neuron and glia cell types after 3 days of differentiation containing approximately 10 and 20 % neurons for human and rat, respectively (Moors et al., 2009, Baumann et al., 2014). Thus, the different cell types might have different sensitivities towards VPA toxicity and the observed 10% increase in apoptosis due to 300 µM VPA in rat NPC could be responsible for selective neuronal loss and not changing the overall viability of the cultures. VPA-induced apoptosis was described as one possible mechanism for the DNT potential of VPA (Ornoy, 2009) caused by its ability to produce reactive oxygen species (ROS; Chang and Abbott, 2006). We showed that this mechanism is indeed responsible for the VPA-dependent neuronal cell death in differentiated rat NPC as the caspase-3 inhibitor DEVD as well as the ROS-scavenger Trolox completely prohibited the VPA-induced loss of β(III)-tubulin$^+$ neurons after 3 days of differentiation (Fig. 4B, G-K). That HDACi is not involved in VPA action on rat neurogenesis is indicated by two lines of evidence: HATi by anacardic acid was not able to prevent VPA-induced reduction in β(III)-tubulin$^+$ neurons (Fig. 4B) and the Notch-dependent, deacetylase Sirt-1-regulated, pro-neural gene *Ascl1* (rev. in Libert et al., 2008) was not affected by VPA in differentiating rat NPC (Fig. 4C). Inhibition of the deacetylase Sirt-1 by VPA has so far only been shown in tumor cells (Hajji et al., 2010), yet the critical role of *Ascl1* for neuronal differentiation in rodents is well established (Ishibashi et al., 1995, Kageyama et al., 1995). In contrast to rat NPC, VPA did not produce neuronal or over all cell death by ROS-induced apoptosis in human NPC, even at a 10-fold higher concentration, because neither the caspase-3 inhibitor DEVD nor the antioxidant trolox antagonized VPA-induced reduction in β(III)-tubulin$^+$ neurons or general cell death (Fig. 4A,D,F,H,J; Supplementary Fig. 2). However, anacardic acid rescued the VPA-dependent

reduction in β(III)-tubulin$^+$ neurons and antagonized the VPA-triggered reduction in *Ascl1* expression (Fig. 4B,C) despite no change in overall reduced cytotoxicity (Fig. 4 A,L; Supplementary Fig. 2). These human data show that VPA-induced loss in β(III)-tubulin$^+$ neurons after 3 days of differentiation is not due to neuronal cell death, but rather caused by epigenetic regulation of neuronal differentiation. However, at these high VPA concentrations cell death of differentiated cells other than young neurons due to so far unknown mechanisms seems to be an independent reason for VPA toxicity.

One reason for these striking species differences in VPA effects on human and rat NPC could root in different metabolism of the parent compound VPA into potentially toxic metabolites (Kiang et al., 2010). Gas chromatography – mass spectrometry (GC-MS) analyses of VPA in these cultures clearly showed that NPC have the ability to metabolize VPA into 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, E-2-ene-VPA and 4-keto-VPA (Fig. 5, Supplementary Figure 3,4). Generation of these metabolites suggest enzyme activities of cytochrome P450 (CYP2C19 and CYP2A6/B6; Kiang et al., 2006). However, there is absolutely no species difference between human and rat NPC concerning VPA metabolism that could explain the specific induction of neuronal apoptosis in rat NPC.

ROS-induced apoptosis is mediated by mitochondrial calcium (Ca^{2+}) release (Annunziato et al., 2003). Therefore, our next hypothesis was that rat NPC might be more sensitive towards VPA-generated ROS because they might differentially express mitochondria-/apoptosis-related proteins in comparison to human NPC. In favor of our hypothesis we found the mitochondrial adenine nucleotide translocator 1 or ADP/ATP translocase Ant1, which is part of the protein complex forming the mitochondrial permeability transition pore (mtPTP; Zoratti and Szabo, 1995, Marzo et al., 1998, Belzacq et al., 2002), significantly higher expressed in rat than in human NPC (expressed in rat 14-fold compared to human NPC; Fig. 6A). Ant1 seems to converge a variety of pro-apoptotic stimuli for execution of apoptosis by increasing mitochondrial membrane permeability (Marzo et al., 1998, Belzacq et al., 2002). *Ant1*-deficient hippocampal and cortical neurons are significantly more resistant towards excitotoxic neuronal death (Lee et al., 2009). ROS production is involved in apoptotic neuronal cell death due to excitotoxicity (Rego and Oliveira, 2003) making Ant1 a probable candidate for VPA-induced, ROS-dependent neuronal death of differentiated NPC in this study. And indeed, similar to DEVD or trolox (Fig. 4B), a knockdown of Ant1 in rat NPC by around 40% (Fig. 6B) abolishes the VPA-dependent loss in β(III)-tubulin$^+$ neurons after 3 days of differentiation (Fig. 6C, E-H). These data strongly indicate that differentiated human NPC are not as sensitive towards ROS-induced apoptosis as their rat counterparts because they express the mtPTP protein Ant1 >10-fold less than the rat cells. This species-specific Ant1 protein expression is reflected in the mRNA abundance of human and rat NPC with significantly higher expression in the latter (Fig. 6D). The relevance of these findings for

humans *in vivo* is indicated by the approximately 11-fold lower expression of Ant1 mRNA in human fetal brain compared to rat brain (Fig. 6D).
Taken together, we generated human- and rat-specific VPA fingerprints for neurodevelopmental endpoints by using the neurosphere assay (Fig. 7).

Discussion

There is an urgent need for alternative methods predicting DNT of chemicals because current *in vivo* tests are by far too resource-intensive and the brain as an important socioeconomic factor for societal well-being needs protection during its developmental process (Grandjean and Landrigan, 2014, Trasande et al., 2015, Crofton et al., 2011, Bal-Price et al., 2015). We propose neurospheres as part of an alternative testing battery to assess compound hazards for neurodevelopmental toxicity. With this case study we support the importance of human-based DNT testing, show that VPA interferes with pathways that possess relevance for neurodevelopment in a species-specific manner, and propose a multi-species fingerprint approach for neurodevelopmental key event evaluation that predicts, in combination with internal exposure data, risk for DNT.

Molecular equipment of the human developing brain seems to contain unique features amongst the animal kingdom (Somel et al., 2011, Zhang et al., 2011). This notion is supported by comparison of proteomes across primary NPC from rodents and humans (Fig. 1). As demonstrated by the PCA analyses, where proteins of the three different species cluster individually, the GO-Term analyses for biological processes and molecular functions clearly show that differences between human and rodents are larger than distinctions between rat and mouse (Suppl. Tab. 2). GO-Terms of basic neurodevelopmental processes like NPC proliferation, migration and differentiation, that are actually performed by proliferating and differentiating neurospheres in culture, are reflected in transcriptome analyses of these cells across the three different species (data not shown). However, these do not appear as differentially enriched in the proteome analyses across species. This makes sense, as basic molecular machineries for e.g. NPC cell cycling or generation of neurons are conserved cellular functions. Interestingly, GO-Terms that differed with high statistical significance were mainly attributed to mitochondrial function and cell metabolism (Suppl. Tab. 2) suggesting that compounds interfering with these cellular/molecular tasks might disclose quantitative differences in effects across species. Because this observation is rather general, a case study seemed necessary that supports this proposition by proof-of-concept.

Most environmental chemicals and also some pharmaceuticals are promiscuous with regard to their MOA (Judson et al., 2010, Burgess-Herbert and Euling, 2013). In addition, cells of different differentiation stages and different cell types as they co-exist in the developing brain exert distinct sensitivities towards pathway modulation (Rice and Barone Jr, 2000). These are part of the reasons why molecular MOA for DNT compounds are largely unknown (Bal-Price et al., 2015) making it extremely difficult to validate an *in vitro* assay for its neurodevelopmental scientific relevance. Tallying to the lack of knowledge on DNT compounds' MOA, unavailability of data for human DNT *in vivo* effects of substances makes assay validation by testing a large chemical test set unfeasible for DNT. One solution for this objective is the thorough comparison of substance effects including their molecular mechanisms across species providing the opportunity to compare human *in vitro* to rodent *in vitro* and ultimately rodent *in vivo* data according to the parallelogram approach (Kienhuis et al., 2009, Waters and Nolan, 1994). Once enough information on comparative functional neurodevelopmental pathway effects is assembled, computational modeling should facilitate DNT hazard and risk assessment by employing MOA-based prediction models in the future (Burgess-Herbert and Euling, 2013). One substance useful for an initial proof-of-principle case study for this proposed concept is the antiepileptic drug and human relatively data-rich DNT compound valproic acid (VPA; Ornoy, 2009). Adverse effects of VPA are attributed to inhibition of histone deacetylases (HDACi; Phiel et al., 2001) or generation of oxidative stress (Verrotti et al., 2008). The latter makes it a worthwhile candidate for addressing species differences in NPC responses due to distinct mitochondrial protein expression as discussed above.

VPA reduces human and rat NPC proliferation and neuronal differentiation and induces apoptosis in differentiated NPC in the neurosphere assay *in vitro* (Baumann et al., 2015b; Fig. 2 A&B, Fig. 3). Time-wise the neurosphere cultures reflect the 2nd trimester of human gestation/rat PND5 (Fig. 1A). The outcomes during this developmental time are clearly distinguishable from adversities on the developing nervous system during embryonic development, where VPA exposure causes increased proliferation (Go et al., 2012), excessive neuronal differentiation (Go et al., 2012, Yu et al., 2009) as well as neural tube defects resulting in spina bifida in humans (Bjerkedal et al., 1982, Dalens et al., 1980). That opposite VPA effects on 2nd trimester human and PND5 rat NPC are indeed due to developmental timing is supported by comparison of consequences of pre- and postnatally VPA-treated rodents, which also produce contrary cell biological adverse outcomes of this pharmaceutical *in vivo* (Yochum et al., 2010, Go et al., 2012, Foti et al., 2013).

Comparing timing-matched rat and human NPC responses towards VPA it is obvious that dramatic species differences are only observed in differentiating NPC (species differences in EC_{50} values for inhibition of neuronal differentiation approx. 10-fold, Fig. 3), while EC_{50} values

for VPA-dependent inhibition of NPC proliferation differ only approximately 2-fold between species (Fig. 2). Thus, species-specific molecular mechanisms are suspected to trigger anti-proliferative and anti-differentiating VPA effects.

That HDACi abilities of VPA contribute to inhibition of mouse NPC proliferation was suspected in a comparative *in vivo-ex vivo-in vitro* study (Foti et al., 2013). We confirm their *in vivo* BrdU and *ex vivo-in vitro* sphere forming assays in the mouse by BrdU incorporation evaluations *in vitro* with human and rat neurospheres. Furthermore, we extend the data by showing that in rat and human NPC VPA reduces proliferation by HDACi because inhibition of the HDAC counter player histone acetylase (HAT) by anacardic acid (AA; Eliseeva et al., 2007) completely antagonizes the VPA-dependent anti-proliferative action accompanied by VPA-dependent induction and AA antagonization of the cell cycle inhibitor $p21^{Cip/WAF1}$ mRNA expression. Cell cycle effects caused by HDAC-dependent $p21^{Cip/WAF1}$ over-expression were seen *in vitro* and *in vivo* earlier (rev. in Gartel et al., 1996). That indeed HDACi is the VPA MOA for inhibition of NPC proliferation is supported by the findings that sodium butyrate, a well-known HDAC inhibitor, but not valpromide, an anti-epileptic drug without HDAC activity (Gurvich et al., 2004), inhibits NPC proliferation (Suppl. Fig. 5). Comparative mRNA and proteome analyses revealed a 2-fold higher expression of HDAC1, one of the main HDACs targeted by VPA (Gurvich et al., 2004), in rat compared to human NPC. Most importantly, this mRNA expression pattern is found in human and rat fetal brain samples from equivalent developmental stages (Fig. 2) thus translating the species difference from *in vitro* to *in vivo* and supporting human relevance.

Similar to NPC proliferation, VPA-dependent neuronal differentiation of human NPC is also reduced via HDACi, yet at concentrations exceeding therapeutic relevance (EC_{50} value = 3177 µM; Fig. 3 & 4A). At these concentrations VPA seems to exert cell type-specific effects on neurons and glia cells. HDACi activity of VPA decreases neuronal differentiation because the VPA-triggered decrease in number of neurons/total nuclei is antagonized by AA and accompanied by a reduction in expression of the pro-neural, HDAC-regulated gene Hash-1 (human homolog of rodent Mash-1, gene name Ascl-1; Libert et al., 2008, Nieto et al., 2001, Ishibashi et al., 1995). The HDAC inhibitor sodium butyrate also reduces neuronal differentiation, while the antiepileptic drug valpromide, which exerts no HDAC activity, does not interfere with neurogenesis (Suppl. Fig. 6). In glia cells, which account for the majority of differentiated NPC in the migration area (Moors et al., 2009, Baumann et al., 2014), the same VPA concentrations cause cell death in an HDACi-independent manner as loss in viability is not antagonized by AA. Caspase inhibition by DEVD or ROS scavenging by Trolox does also not rescue glia cells from VPA-induced cell death (Suppl. Fig. 2) suggesting an HDAC- and ROS-independent mechanism. In contrast to effects in human NPC, VPA causes ROS- and caspase-dependent neuronal apoptotic cell death in differentiated rat NPC

completely independent of HDACi (Fig. 3 B+C, 4B). VPA-induced neuronal apoptosis was seen earlier in rodents *in vivo* (Yochum et al., 2010) and *in vitro* (Wang et al., 2011), yet its pro- or anti-apoptotic effects seem to depend on developmental timing (Go et al., 2011).

These species-specific VPA MOA on neuronal differentiation/young neurons is not due to species-dependent VPA metabolism because VPA metabolites produced after 48 hrs in culture are very similar between human and rat NPC (Fig. 5). Moreover, metabolite pattern of differentiated NPC is very similar to proliferating NPC (Suppl. Fig. 4), where VPA did not trigger apoptosis. In search of factors that might determine human NPC low and rat NPC high sensitivity towards the apoptosis-inducing stimuli of VPA, we extracted two possible explanations out of the proteome analyses: the cellular antioxidative defense and the mitochondrial permeability transition pore (mtPTP) complex assembly, both known players in apoptosis initiation and execution (Chandra et al., 2000, Crompton, 1999).

First, proteins involved in protection against ROS are clearly higher expressed in human than in rat NPC (Suppl. Fig. 7). These proteins are mainly associated with glutathione-related cellular metabolism, one of the main lines of antioxidant defense in brain. In rat liver and brain VPA induces oxidative stress (Hishida and Nau, 1998, Al-Amin et al., 2015) and, at least in liver, glutathione is an integral part of the protection against VPA-induced toxicity (Tong et al., 2005). VPA-generated reactive metabolites and/or ROS alter glutathione homeostasis in humans and animal models (Cotariu et al., 1990, Graf et al., 1998, Cengiz et al., 2000) emphasizing the involvement of glutathione in VPA metabolism also in humans. In the developing mouse brain elevation of VPA-reduced glutathione levels by co-exposure with an antioxidant correlates with improved VPA-altered animal behavior (Pragnya et al., 2014) and transgenic glutathione S-transferase M1-deficiency exacerbates VPA-induced apoptosis in postnatal female mouse brains (Yochum et al., 2010) suggesting that in rodents VPA-dependent ROS generation contributes to its DNT properties. These data strengthen the postulation that increased cellular antioxidative defense, as seen in human compared to rat NPC (Suppl. Fig. 7) or experimentally achieved by treatment of rat NPC with the antioxidant Trolox (Fig. 4B), protect against VPA-induced neuronal apoptosis.

The VPA-dependent, pro-apoptotic phenotype of differentiated rat NPC, which is favored by a rat species-specific sub-optimal antioxidative line of defense (Suppl. Fig. 7), is further triggered by a ‘sensitive’ composition of the mtPTP protein complex. A crucial player in this complex is the mitochondrial carrier protein adenine nucleotide translocator (ANT; rev. in Belzacq et al., 2002), the most abundant protein in the inner mitochondrial matrix (Klingenberg, 1993). One of the four ANT isoforms found in mammals, ANT1, is present in the brain and is essential for cellular energy metabolism by exchanging cellular ADP for ATP generated inside the mitochondrial matrix (rev. in Chevrollier et al., 2011). As a bi-functional

protein, ANT1 can undergo a functional switch into a pro-apoptotic player of the mPTP mediating flux of small molecules of MM < 1500 Da and thus disruption of mitochondrial homeostasis with loss of mitochondrial membrane potential, mitochondrial swelling, and at last rupture of the outer mitochondrial membrane with release of soluble pro-apoptotic proteins from the intermembrane space (Jang et al., 2008, rev. in Belzacq et al., 2002). This pro-apoptotic, pore-forming stage of ANT1 can be triggered by endogenous molecules like Bax and Bid as well as by pro-oxidative xenobiotics like arsenite, peroxinitrite or tert-butylhydroperoxide (rev. in Belzacq et al., 2002). Here we show that ANT1 mRNA and protein is highly expressed in differentiated rat NPC compared to human counterparts (Fig. 6A,D), that knockdown of rat NPC ANT1 seems to protect neurons from VPA-induced apoptosis (Fig. 6B,C) and that there is strong indication that differential expression of this pro-apoptotic protein across these two species holds true for developing brain *in vivo* (Fig. 6D). Taken together, these data provide one example that molecular composition of NPC determines toxicodynamics of compounds in a species-specific manner. More compounds have given species-specific sensitivities of NPC towards a variety of compounds earlier (Gassmann et al., 2010, Baumann et al., 2015b).

When performing risk assessment, *in vitro* 'fingerprints' of altered neurodevelopmental key events, like the ones presented in this study in combination with e.g. other endpoints indispensable for brain development like synaptogenesis and neuronal network activity or molecular biomarkers, will be valuable measures for DNT hazard assessment. As a proof-of-principle, a similar approach not related to DNT and for a much greater number of compounds assayed complex biological pathways in primary human cells and identified potential chemical targets and molecular mechanisms useful for elucidating AOPs (Kleinstreuer et al., 2014). Due to the molecular equipment of NPC reflecting the *in vivo* situation well for the molecules evaluated in this study (Fig. 2H & 6D), we suggest that internal exposure of fetal rat – or, in case available, human - brains derived by experimental measures or physiologically based pharmacokinetic (PBPK) modeling (Croom et al., 2015) could possibly help shaping DNT risk assessment for the 21st century e.g. within the AOP concept (Fig. 7; Baumann et al., 2015b, Sturla et al., 2014). Scientific, pathway-based validation of *in vitro* systems like the neurosphere assay towards human *in vivo* data as well as compound testing are urgently needed to define the biological application domain of such methods. Knowing strengths and weaknesses of the neurosphere assay will improve certainty in *in vitro* results for either risk assessment or prioritization of compounds as a result of a screening effort generating neurosphere 'fingerprints'.

Taken together, this case study with the antiepileptic drug VPA provided a proof-of-principle for using primary human and rat neurospheres as part of a DNT screening effort to predict alterations of signaling pathways and cellular functions in a species-specific manner.

Molecular equipment found in NPC *in vitro* was shown to have relevance to humans *in vivo*. Thus, this comparative neurosphere assay as part of a larger key event screening battery could be applied for improving human DNT risk assessment for example within the AOP concept.

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Figure Legends

Figure 1: Human and rodent NPC reveal molecular species differences. a. Timeline of neurodevelopment for human and rodent NPC generation. **b. and c.** Principal component analysis of the proteome of proliferating (b.) and differentiating (c.) human, mouse and rat neurospheres. For the quantitative analysis of proteins expressed by proliferating neurospheres, a label-free approach using signal intensities in the mass spectrometer was employed. The principal component analysis shows the distribution of the proteomic profile of 5 to 6 replicates per species.

Figure 2: Effects of VPA on human and rat NPC proliferation. **a. and b.** Proliferation and viability were assessed by testing 7 concentrations of VPA in human (a.) and rat (b.) neurospheres. Therefore, 4 floating spheres per exposure group were plated one sphere per 96-well for 3 days. At the end of exposure time, proliferation was assessed by BrdU Assay and viability was monitored by Alamar Blue Assay. Concentration response curves are shown for human and rat neurospheres. Values are given as average percentages of solvent control for the endpoints proliferation (BrdU) and viability (Alamar Blue) ± SEM (n = 6-7 independent experiments). Asterisks (proliferation) and crosses (viability) denote significance respect to solvent control ($p < 0.05$). Sigmoidal concentrations response curve fits have been previously published in Baumann et al. (2015b). **c. and d.** Floating human (c.) and rat (d.) neurospheres were pre-incubated with 50 µM anacardic acid for 1 h and afterwards co-incubated with anacardic acid and VPA for 3 days. At the end of exposure time, proliferation was assessed by BrdU assay. Values are given as average percentages of solvent control ± SEM (n = 3-4 independent experiments). Asterisks denote significance ($p < 0.05$). **e. and f.** mRNA expression of $p21^{Cip/WAF1}$ was quantified after co-exposure of human (e.) and rat (f.) neurospheres with anacardic acid and VPA. Therefore, 10 floating spheres per 24-well in triplicates were pre-incubated with 50 µM anacardic acid for 1 h and afterwards co-incubated with anacardic acid and VPA for 12 hrs. Expression of $p21^{Cip/WAF1}$ and β-actin as housekeeping gene was quantified by real-time RT-PCR. Values are given in average fold of solvent control, calculated by delta-delta-CT-method, ± SEM (n = 3-5 independent experiments). Asterisks denote significance ($p < 0.05$). **g.** Protein expression of HDAC1 was determined by proteome analysis of proliferating human and rat neurospheres. Values are given in average normalized protein abundances ± SD (n = 6 replicates). **h.** Expression of HDAC1 in proliferating human and rat neurospheres and brain RNA samples was quantified by real-time RT-PCR. Values are given in average copy numbers per 1000 copies of β-actin ± SEM (n = 3 independent experiments). Asterisks denote significance ($p < 0.05$).

Figure 3: Effects of VPA on the amount of neurons in differentiating human and rat NPC. **a. and b.** The differentiation of NPC to neurons and viability were assessed by testing 7 concentrations of VPA in human (a.) and rat (b.) neurospheres. Therefore, 5 spheres per exposure group were plated in one well of a PDL/laminin coated 8-Well chamber slide for 3 days. At the end of exposure time, viability was monitored by Alamar Blue Assay and neurospheres were fixed, immunocytochemically stained for βIII-tubulin and counterstained with Hoechst. Immunofluorescent pictures were taken and neurons were quantified by manual counting. Sigmoidal concentrations response curve fits are shown for human and rat neurospheres. Values are given as average percentages of solvent control for the endpoints neuronal differentiation (neuronal diff.) and viability (Alamar Blue) ± SEM (n = 3-15 independent experiments). Asterisks (neuronal differentiation) and crosses (viability) denote significance respect to solvent control ($p < 0.05$). Concentrations response curves have been previously published in Baumann et al. (2015b). **c.** The amount of apoptotic cells in differentiating human and rat neurospheres was quantified by Life-Dead-Assay. Therefore, 5 spheres per exposure group were plated in one well of a PDL/laminin coated 8-Well chamber slide for 48 hrs. 30 min prior the end of exposure time, Hoechst and propidium iodide were added, cells were fixed and slides were mounted. Immunofluorescent pictures were taken and apoptotic cells were quantified by manual counting. Values are given as average percentages of control ± SEM (n = 4-13 independent experiments). Asterisks (rat) and hashs (human) denote significance respect to control ($p < 0.05$). **d. to g.** Immunoflourescent pictures of βIII-tubulin (red) and Hoechst (blue) staining for 3 days differentiated human (d.) and rat (f.) neurosphere solvent controls and VPA treated human (3750 µM, e.) and rat (417 µM, g.) neurospheres**. h. to i.** Immunofluorescent pictures of propidium iodide (red) and Hoechst (blue) staining for 48 hrs differentiated human (h.) and rat (j.) neurosphere solvent controls and VPA treated human (3000 µM, i.) and rat (300 µM, k.) neurospheres. Scale bars 100 µm.

Figure 4: Pathway investigations for neuronal loss in differentiating human and rat neurospheres. a. and b. Differentiating human (a.) and rat (b.) neurospheres were co-incubated with VPA and a caspase-3 inhibitor (DEVD), a scavenger of reactive oxygen species (trolox) or a HAT-inhibitor (anacardic acid), respectively. Therefore, 5 spheres per exposure group were plated in one well of a PDL/laminin coated 8-Well chamber slide and pre-incubated with 50 µM DEVD or 50 µM trolox for 2 hrs, or with 1 µM anacardic acid for 1 h and afterwards co-incubated with VPA for 3 days. At the end of exposure time, neurospheres were fixed, immunocytochemically stained for βIII-tubulin and counterstained with Hoechst. Immunofluorescent pictures were taken and neurons were quantified by manual counting. Values are given as average percentages of solvent control ± SEM (n = 3-8 independent experiments). Asterisks denote significance ($p < 0.05$). For displayed bar diagrams, solvent

controls and VPA-solvent co-treatments were pooled, but statistics was performed with the appropriate solvent and VPA controls. **c.** Human and rat neurospheres were differentiated in presence of VPA for 3 days or preincubated with 1µM ancardic acid for 1h and treated with VPA for 3 days afterwards. Therefore, neurospheres were chopped to 0.1 mm and 100 pieces of chopped neurospheres were plated per PDL/laminin coated 24-well in triplicates per exposure group. At the end of exposure time, spheres were harvested for mRNA isolation and cDNA synthesis and expression of Ascl1 and β-actin as housekeeping gene was quantified by quantitative real-time RT-PCR. Values are given in average fold of solvent control, calculated by delta-delta-CT-method, ± SEM (n = 3-4 independent experiments). Asterisks denote significance ($p < 0.05$). **d. to l.** Immunofluorescent pictures of βIII-tubulin (red) and Hoechst (blue) staining for 3 days differentiated human (c., e., g., i. and k.) and rat (d., f., h., j. and l.) neurospheres. Pictures of solvent controls (c. and d.), VPA treated human (2500 µM) and rat (250 µM) neurospheres (e. and f.) and cotreated human and rat neurospheres with DEVD (g. and h.), trolox (i. and j.) and anacardic acid (k. and l.) are shown. Scale bars 100 µm.

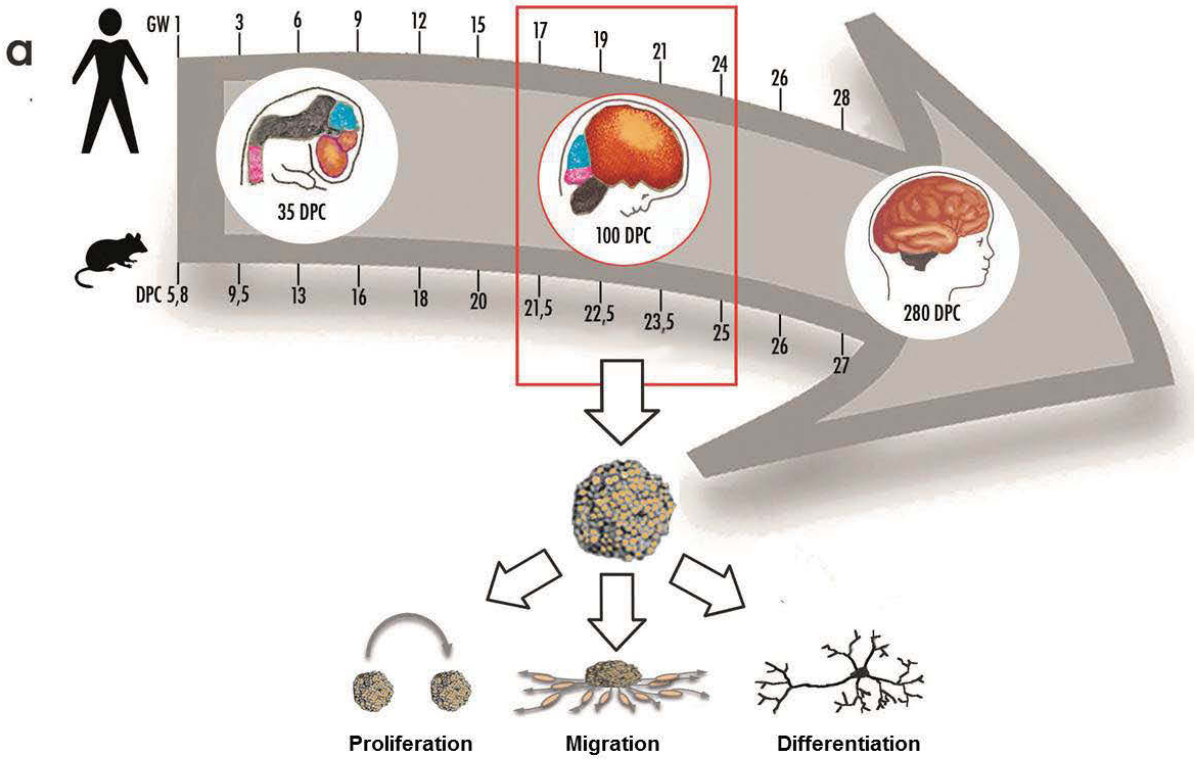
Figure 5: Analysis of VPA metabolites in human and rat neurospheres. Differentiating human and rat neurospheres were treated with 1000 µM VPA for 48 hrs. At the end of exposure time, supernatant was collected and VPA metabolites were quantified using a gas chromatography – mass spectrometry (GC-MS) assay. **a.** Representative extracted ion chromatograms of human neurospheres treated with 1000 µM VPA and respective standards are shown for ene-VPA metabolites and OH-VPA metabolites. For 4-keto-VPA chromatograms, see Supplementary Figure 3. **b.** Concentration of VPA metabolites, values are given in average nanomolar metabolite concentrations ± SD (n = 2-4 independent experiments). For concentrations of VPA metabolites in proliferating human and rat neurospheres, see Supplementary Figure 4.

Figure 6: Analysis of the influence of ANT1 on neuronal sensitivity to VPA in differentiating human and rat neurospheres. a. Protein expression ANT1 was determined by proteome analysis of differentiated human and rat neurospheres. Values are given in average normalized protein abundances ± SD (n = 4-6 replicates). **b.** The expression ANT1 mRNA was knocked down in rat neurospheres. Therefore, rat neurospheres were chopped to 0.1 mm pieces and afterwards transfected with either scrambled siRNA or ANT1 siRNA by nucleofection. 16 hrs after transfection, around 50 small spheres were plated per PDL/laminin coated 24-well in triplicates each. After 72 hrs of differentiation, spheres were harvested for mRNA isolation and cDNA synthesis and expression of ANT1 and β-actin as housekeeping gene was quantified by quantitative real-time RT-PCR. Values are given in average copy numbers of ANT1 per 1000 copies of β-actin, preliminary result of one

experiment is shown. **c.** Rat neurospheres transfected with scrambled siRNA or ANT1 siRNA were exposed to 417 µM VPA for 72 hrs. Therefore, 8 spheres per exposure group were plated in one well of a PDL/laminin coated 8-Well chamber slide. At the end of exposure time, neurospheres were fixed, immunocytochemically stained for βIII-tubulin and counterstained with Hoechst. Immunoflourescent pictures were taken and neurons were quantified by manual counting. Values are given as percentages of control, preliminary result of one experiment is shown. **d.** Expression of ANT1 in 3 days differentiated human and rat neurospheres and brain RNA samples was quantified by quantitative real-time RT-PCR. Values are given in average copy numbers per 1000 copies of β-actin ± SEM (n = 3-4 independent experiments). Asterisk denotes significance ($p < 0.05$). **e. to h.** Immunofluorescent pictures of βIII-tubulin (red) and Hoechst (blue) staining for 3 days differentiated rat neurospheres transfected with scrambled siRNA (e. and g.) or ANT1 siRNA (f. and h). Spheres were treated either with solvent (e. and f.) or 417 µM VPA (g. and h.). Scale bars 100 µm.

Figure 7: New approach of chemical risk assessment using species specific fingerprints. Human and rat neurospheres are exposed towards a chemical and concentration response curves for functional key events of neurodevelopment are recorded, creating species-specific compound fingerprints. By incorporating human and rat internal exposure levels, a regulation of chemical risk with the help of *in vitro* data is feasible. Number of key event measures for fingerprint generation is not known yet.

Figure 1: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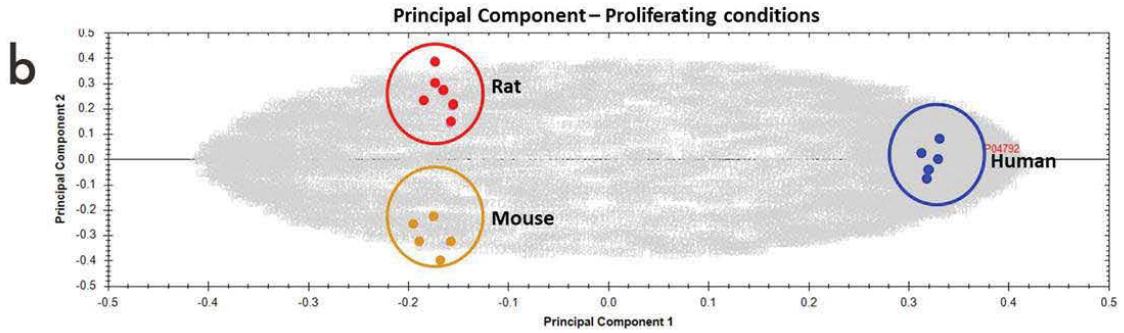
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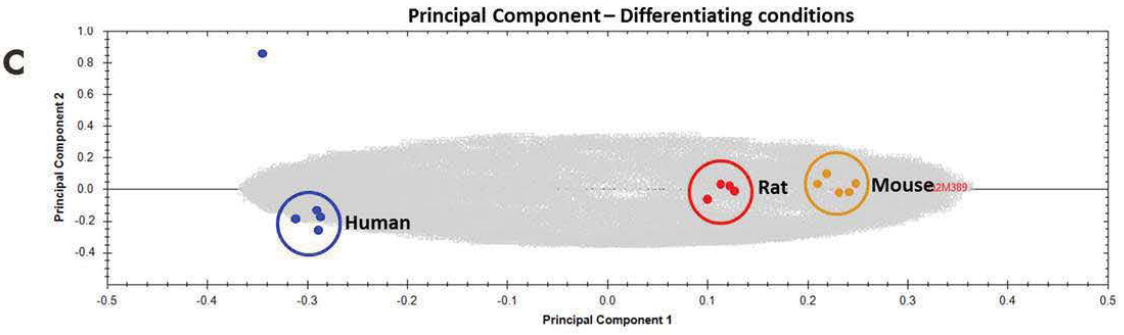
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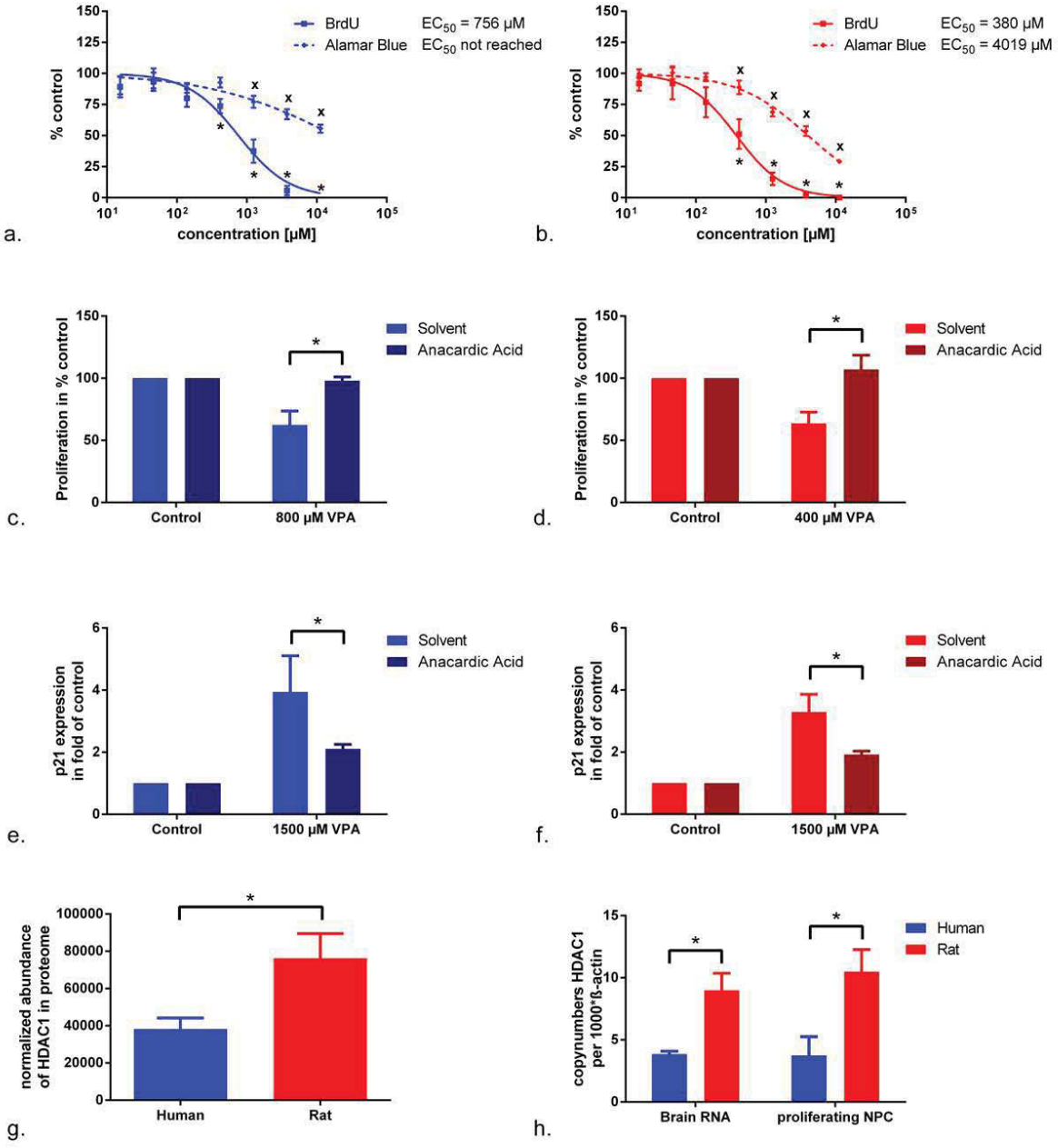
Figure 2: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Proliferation in % control
Solvent
Anacardic Acid
Control
800 µM VPA
d.
Proliferation in % control
Solvent
Anacardic Acid
Control
400 µM VPA
e.
p21 expression in fold of control
Solvent
Anacardic Acid
Control
1500 µM VPA
f.
p21 expression in fold of control
Solvent
Anacardic Acid
Control
1500 µM VPA
g.
normalized abundance of HDAC1 in proteome
Human
Rat
h.
copynumbers HDAC1 per 1000*ß-actin
Human
Rat
Brain RNA
proliferating NPC

Figure 3:

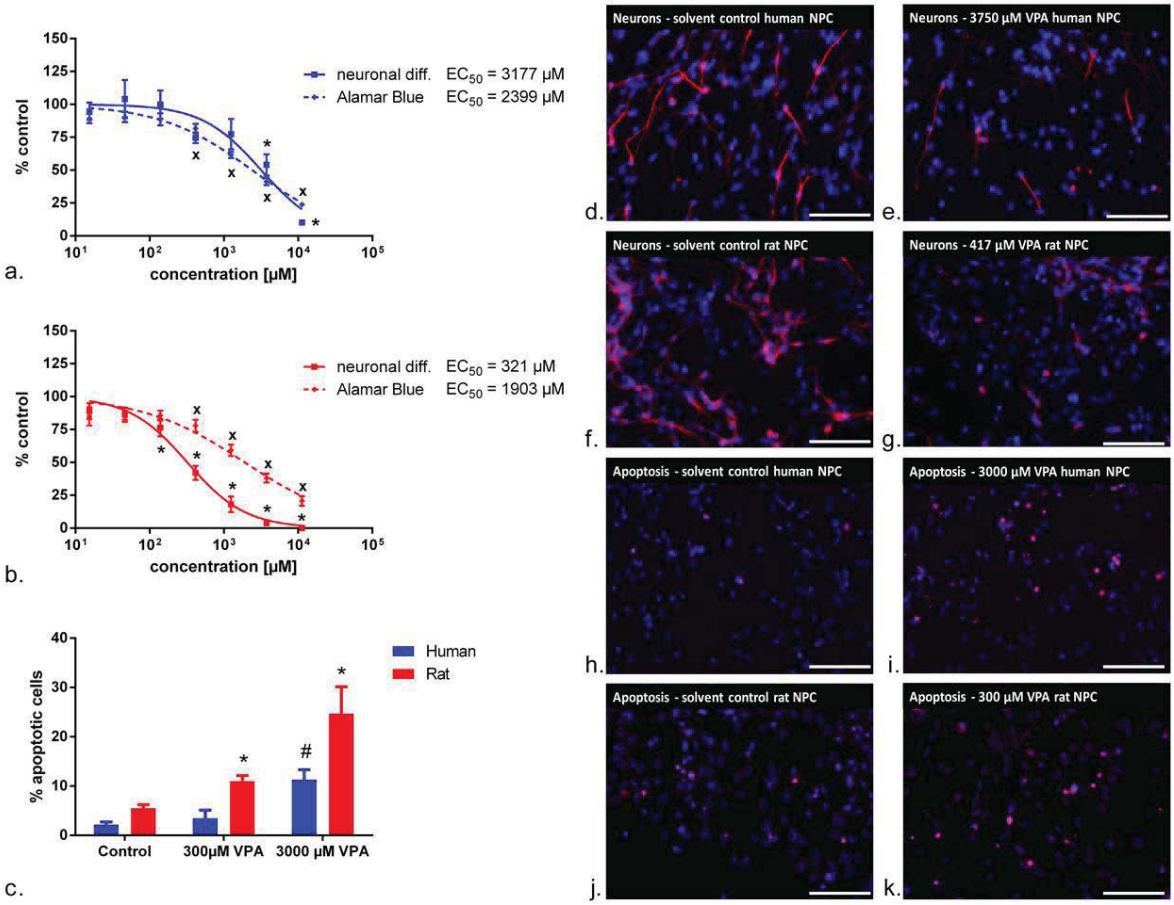

a.
% control
concentration [µM]
neuronal diff. EC50 = 3177 µM
Alamar Blue EC50 = 2399 µM
b.
% control
concentration [µM]
neuronal diff. EC50 = 321 µM
Alamar Blue EC50 = 1903 µM
c.
% apoptotic cells
Human
Rat
Control
300µM VPA
3000 µM VPA
d. Neurons - solvent control human NPC
e. Neurons - 3750 µM VPA human NPC
f. Neurons - solvent control rat NPC
g. Neurons - 417 µM VPA rat NPC
h. Apoptosis - solvent control human NPC
i. Apoptosis - 3000 µM VPA human NPC
j. Apoptosis - solvent control rat NPC
k. Apoptosis - 300 µM VPA rat NPC

Figure 4:

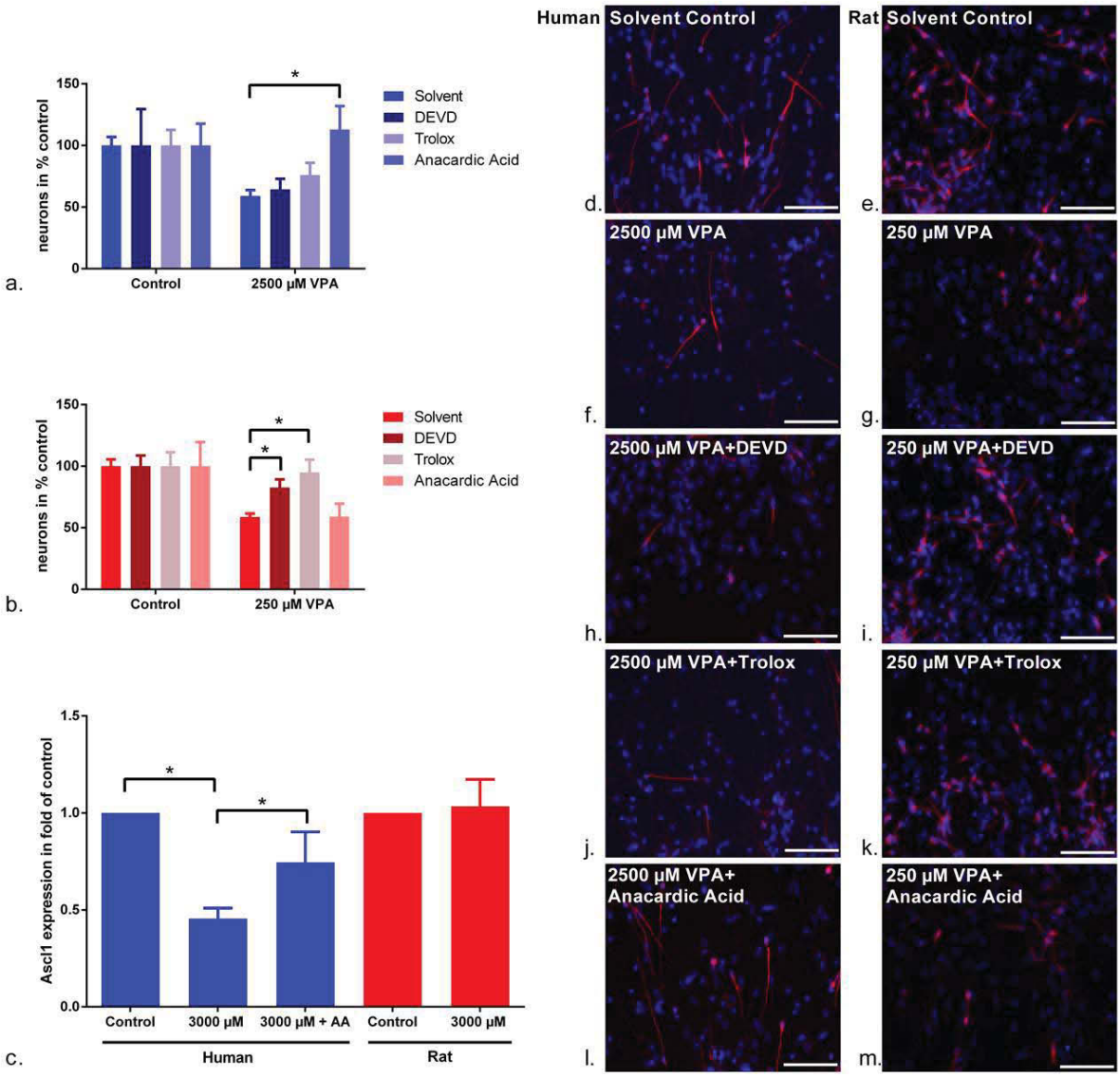
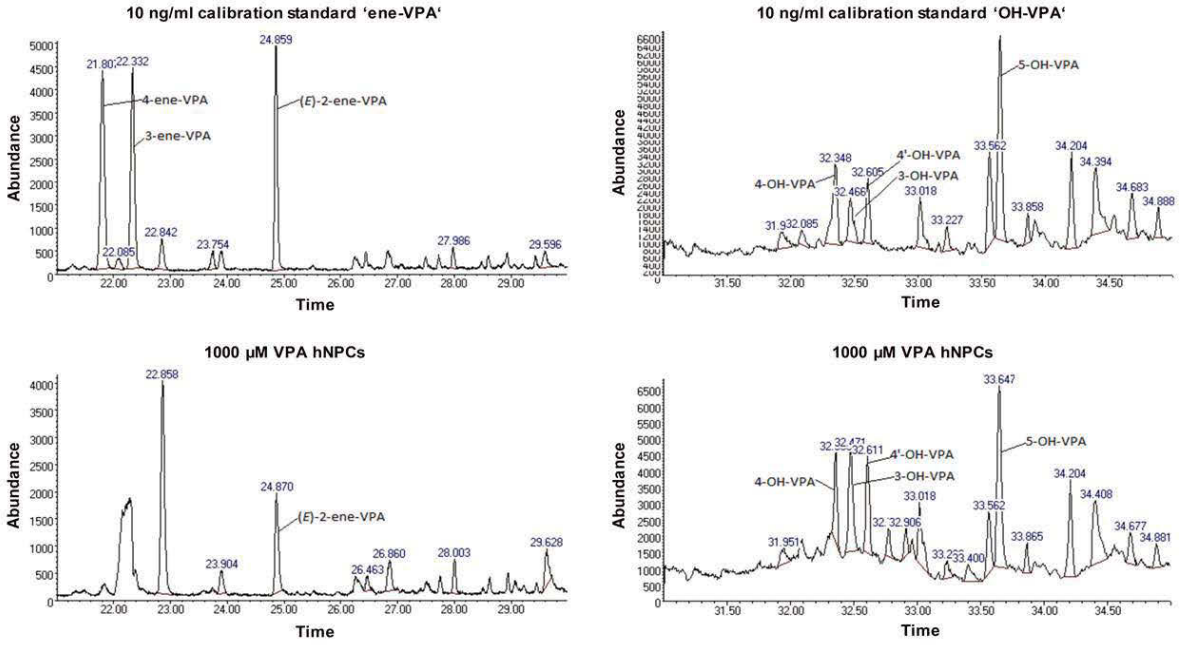
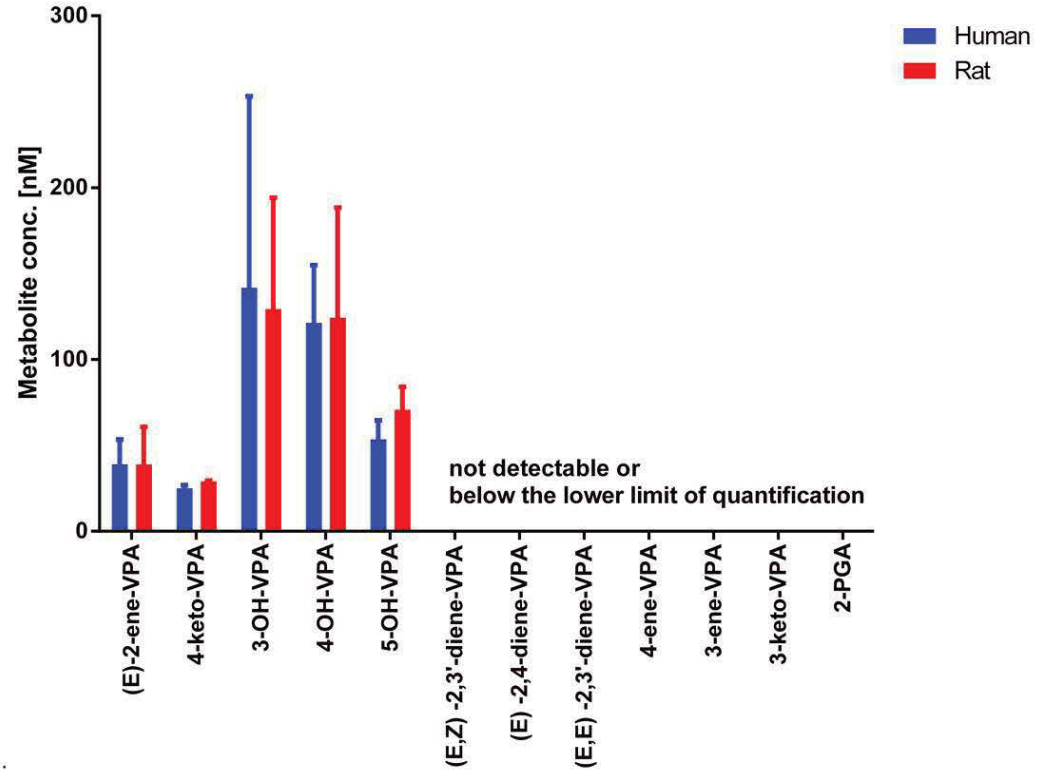

a.
neurons in % control
150
100
50
0
Control
2500 µM VPA
Solvent
DEVD
Trolox
Anacardic Acid
b.
neurons in % control
Control
250 µM VPA
Solvent
DEVD
Trolox
Anacardic Acid
c.
Ascl1 expression in fold of control
1.5
1.0
0.5
0.0
Control
3000 µM
3000 µM + AA
Control
3000 µM
Human
Rat
Human
Solvent Control
Rat
Solvent Control
d.
e.
2500 µM VPA
250 µM VPA
f.
g.
2500 µM VPA+DEVD
250 µM VPA+DEVD
h.
i.
2500 µM VPA+Trolox
250 µM VPA+Trolox
j.
k.
2500 µM VPA+
Anacardic Acid
250 µM VPA+
Anacardic Acid
l.
m.

Figure 5:


10 ng/ml calibration standard 'ene-VPA'
Abundance
Time
21.80
22.085
22.332
22.842
23.754
24.859
27.986
29.596
4-ene-VPA
3-ene-VPA
(E)-2-ene-VPA
10 ng/ml calibration standard 'OH-VPA'
31.932
32.085
32.348
32.466
32.605
33.018
33.227
33.562
33.858
34.204
34.394
34.683
34.888
4-OH-VPA
4'-OH-VPA
3-OH-VPA
5-OH-VPA
1000 µM VPA hNPCs
22.858
23.904
24.870
26.463
26.860
28.003
29.628
(E)-2-ene-VPA
1000 µM VPA hNPCs
31.951
32.32.471
32.611
32.906
33.018
33.233
33.400
33.562
33.647
33.865
34.204
34.408
34.677
34.881
4-OH-VPA
4'-OH-VPA
3-OH-VPA
5-OH-VPA

a.


Human
Rat
Metabolite conc. [nM]
300
200
100
0
not detectable or
below the lower limit of quantification
(E)-2-ene-VPA
4-keto-VPA
3-OH-VPA
4-OH-VPA
5-OH-VPA
(E,Z) -2,3'-diene-VPA
(E) -2,4-diene-VPA
(E,E) -2,3'-diene-VPA
4-ene-VPA
3-ene-VPA
3-keto-VPA
2-PGA

b.

Figure 6:

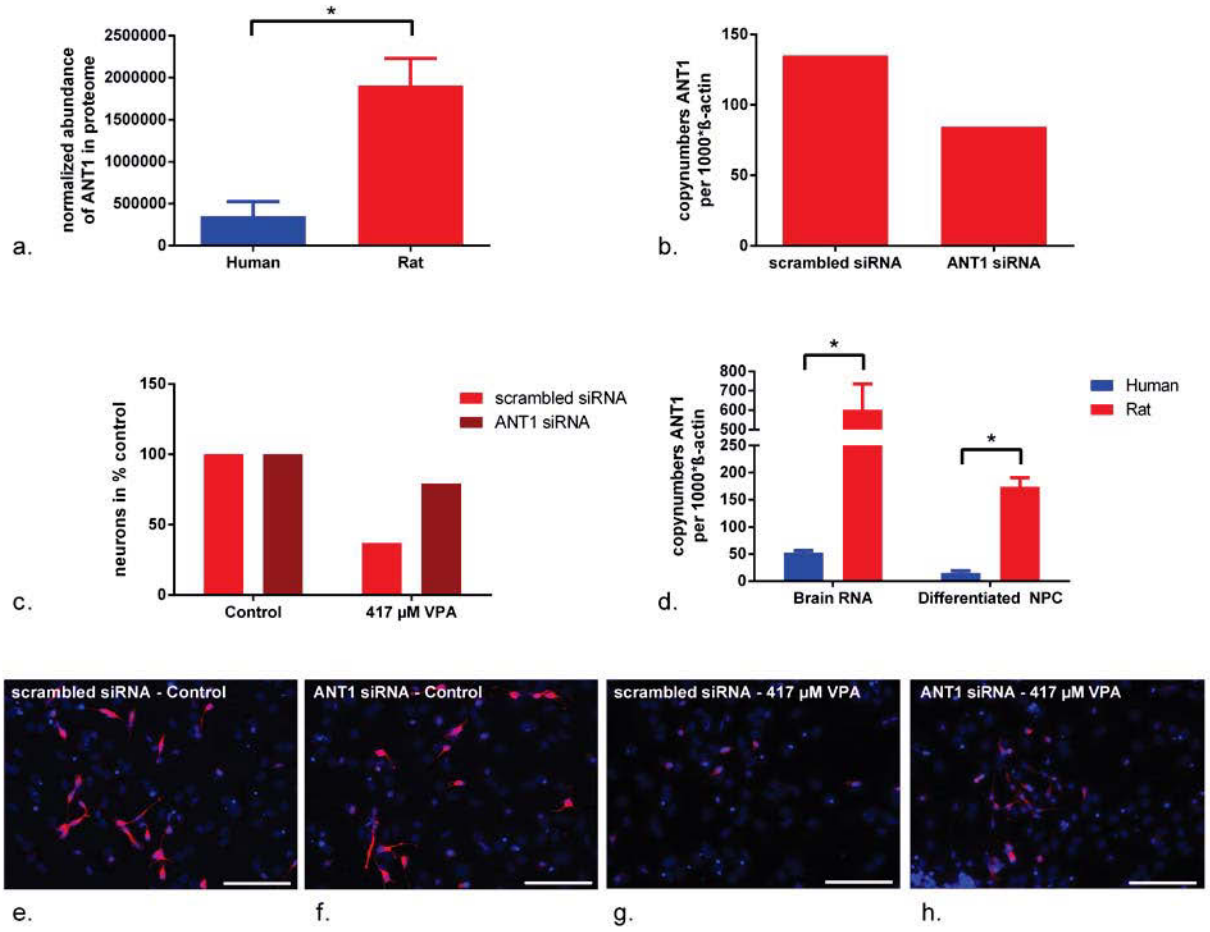
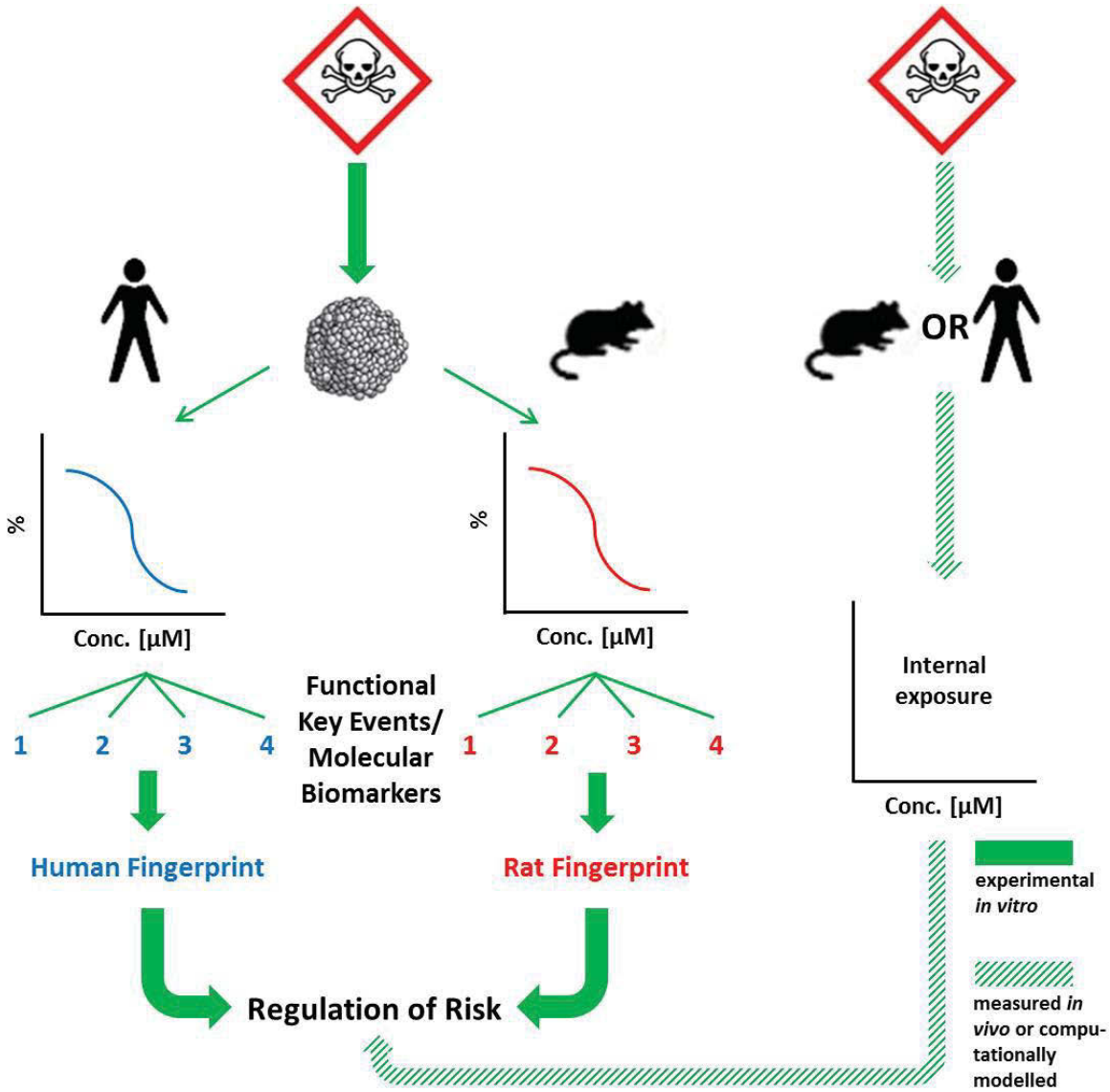

a.
normalized abundance of ANT1 in proteome
2500000
2000000
1500000
1000000
500000
0
*
Human
Rat
b.
copynumbers ANT1 per 1000*ß-actin
150
100
50
0
scrambled siRNA
ANT1 siRNA
c.
neurons in % control
150
100
50
0
scrambled siRNA
ANT1 siRNA
Control
417 µM VPA
d.
copynumbers ANT1 per 1000*ß-actin
800
700
600
500
250
200
150
100
50
0
*
*
Human
Rat
Brain RNA
Differentiated NPC
scrambled siRNA - Control
ANT1 siRNA - Control
scrambled siRNA - 417 µM VPA
ANT1 siRNA - 417 µM VPA
e.
f.
g.
h.

Figure 7:


OR
%
Conc. [µM]
%
Conc. [µM]
Internal exposure
Conc. [µM]
1
2
3
4
Functional Key Events/ Molecular Biomarkers
1
2
3
4
Human Fingerprint
Rat Fingerprint
Regulation of Risk
experimental in vitro
measured in vivo or computationally modelled

Electronic supplementary material

21st Century Risk Assessment: case study for species-specific hazard characterization of valproic acid

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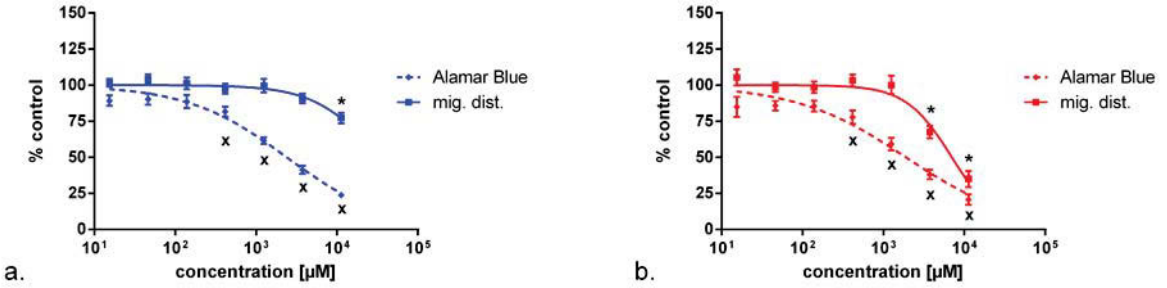
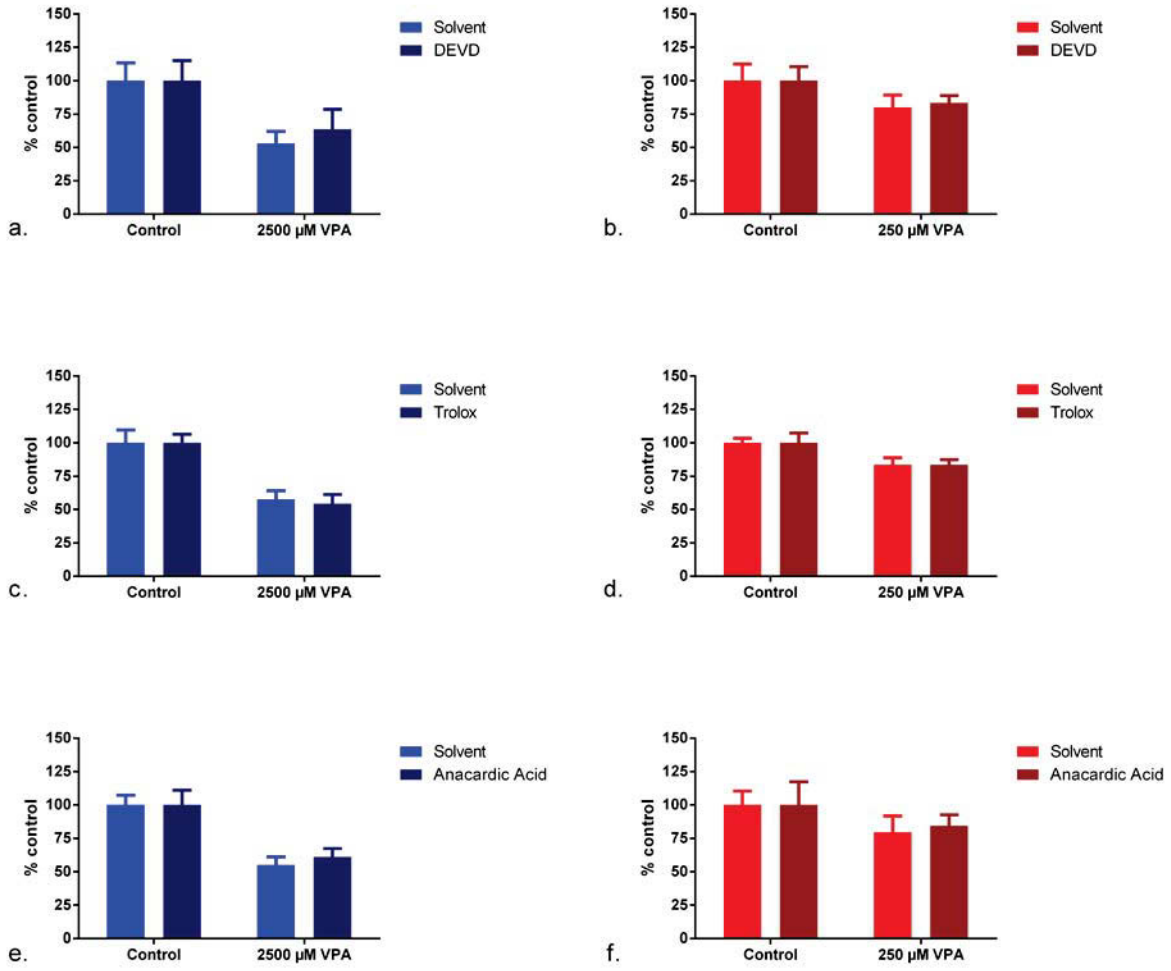
Figure S1:



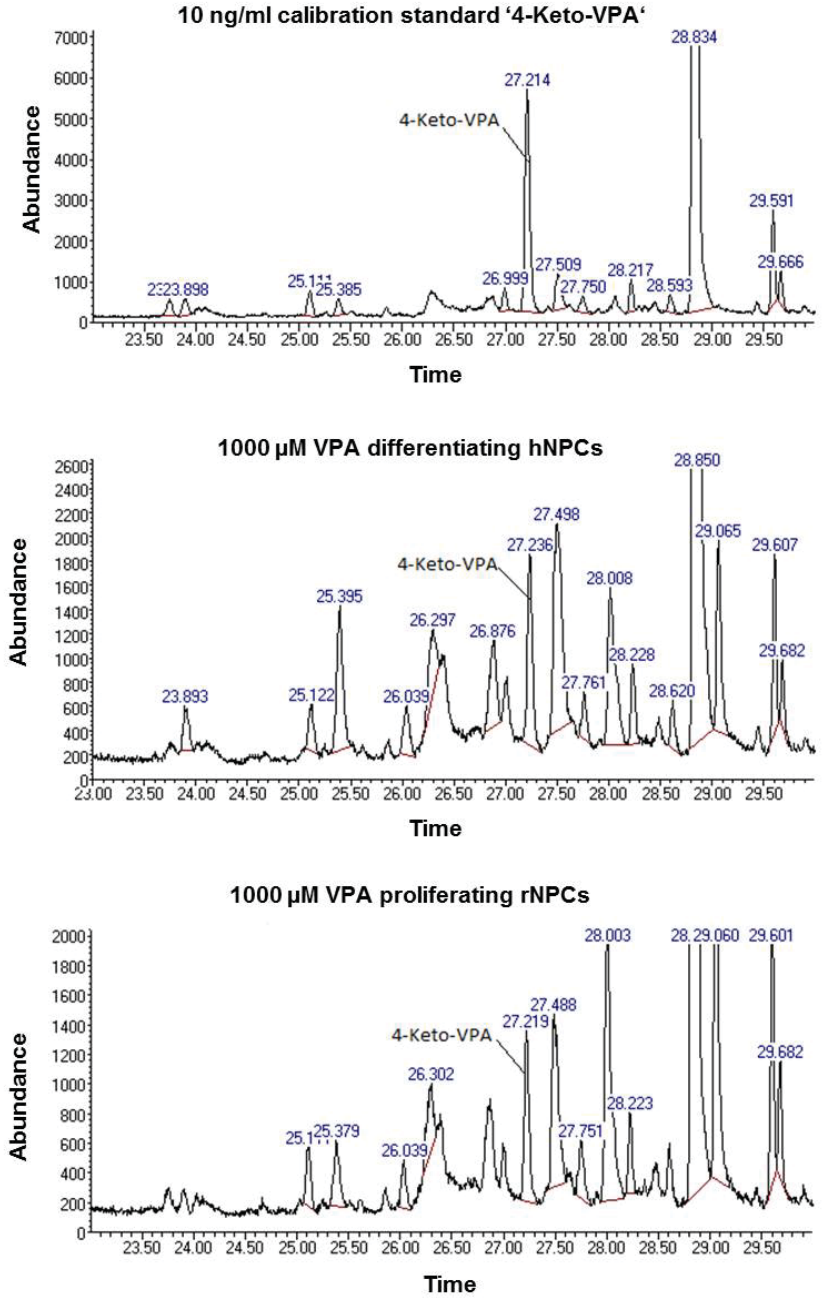
Supplementary Figure 1 NEU: Effects of VPA on human and rat neurosphere migration. Migration and viability were assessed by testing 7 concentrations of VPA in human (a.) and rat (b.) neurospheres. Therefore, 5 spheres per exposure group were plated in one well of a PDL/laminin coated 8-Well chamber slide for 3 days. At the end of exposure time, phase contrast pictures were taken for migration distance measurement and viability was monitored by Alamar Blue Assay. Concentration response curves are shown for human and rat neurospheres. Values are given as average percentages of solvent control for the endpoints migration (mig. dist.) and viability (Alamar Blue) ± SEM (n = 9-15 independent experiments). Asterisks (migration) and crosses (viability) denote significance respect to solvent control ($p < 0.05$).

Figure S2:



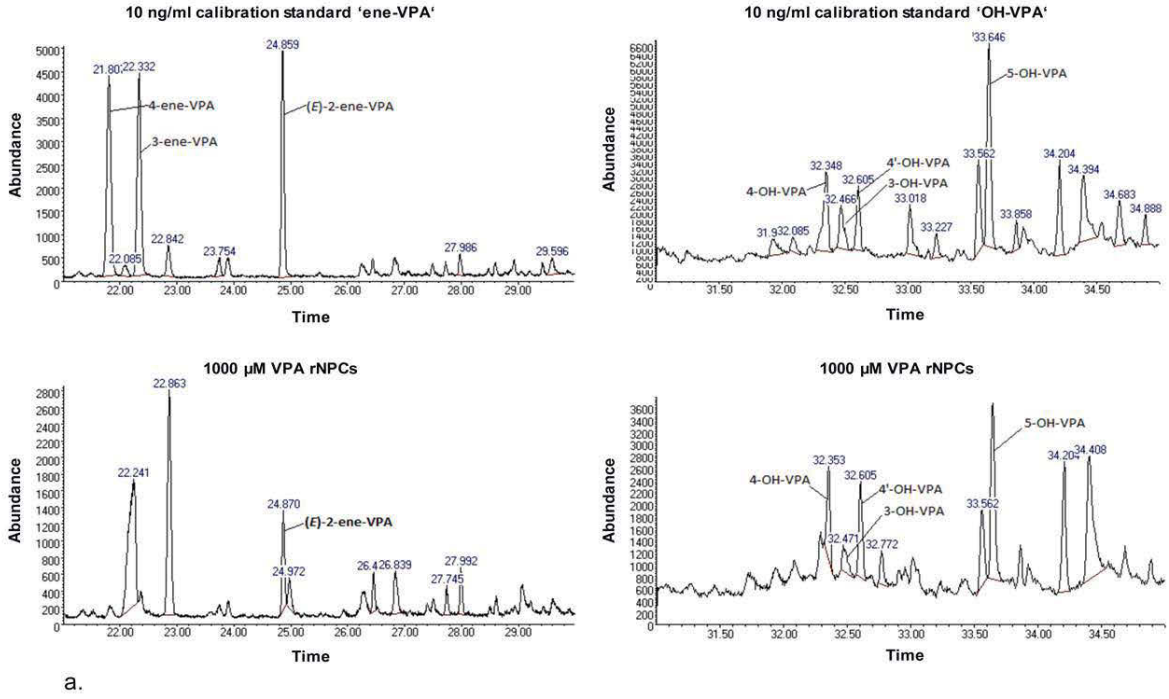
Supplementary Figure 2: Viability data for inhibitor investigations of differentiating human and rat neurospheres. Differentiating human (a., c. and e.) and rat (b., d. and f.) neurospheres were co-incubated with VPA and a caspase-3 inhibitor (DEVD), a scavenger of reactive oxygen species (trolox) or anacardic acid, respectively. Therefore, 5 spheres per exposure group were plated in one well of a PDL/laminin coated 8-Well chamber slide and pre-incubated with DEVD or trolox for 2 hrs, or with anacardic acid for 1 h and afterwards co-incubated with VPA for 3 days. At the end of exposure time, viability of neurospheres was assessed by Alamar Blue assay. Values are given as average percentages of solvent control ± SEM (n = 3-8 independent experiments).

Figure S3:

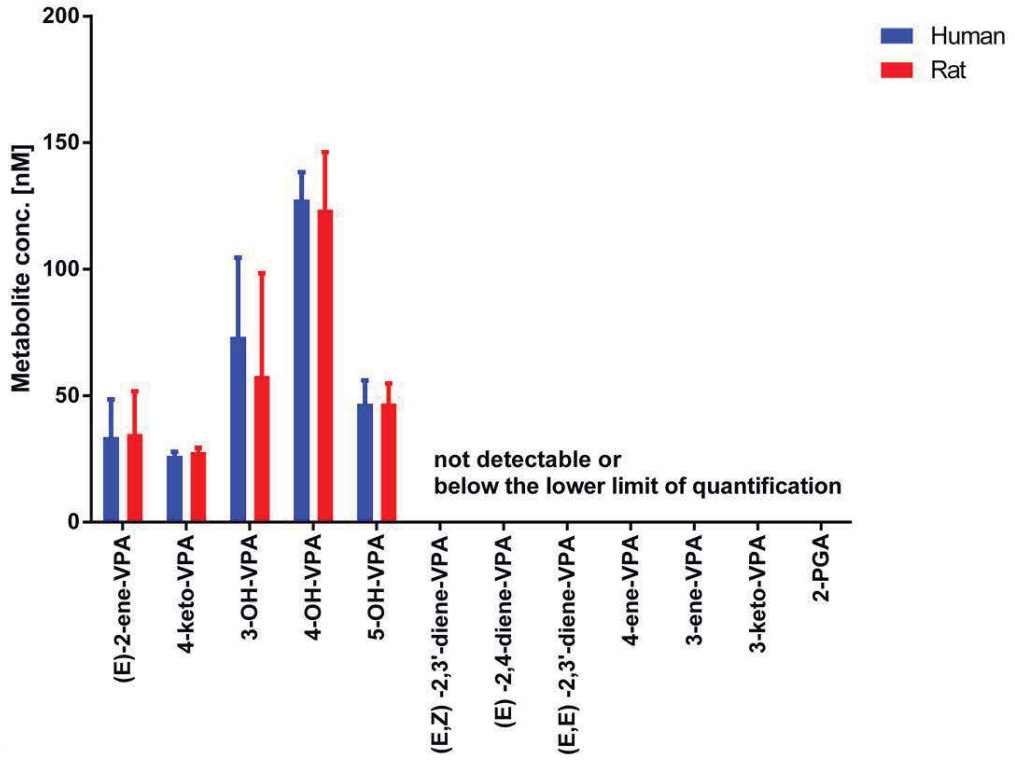


Supplementary Figure 3: Analysis of 4-keto-VPA in human and rat neurospheres. Proliferating and differentiating human and rat neurospheres were treated with 1000 µM VPA for 48 hrs. At the end of exposure time, supernatant was collected and VPA metabolites were quantified using a gas chromatography – mass spectrometry (GC-MS) assay. Representative extracted ion chromatograms of differentiating human neurospheres and proliferating rat neurospheres treated with 1000 µM VPA with respective standard are shown for 4-keto-VPA metabolites.

Figure S4:



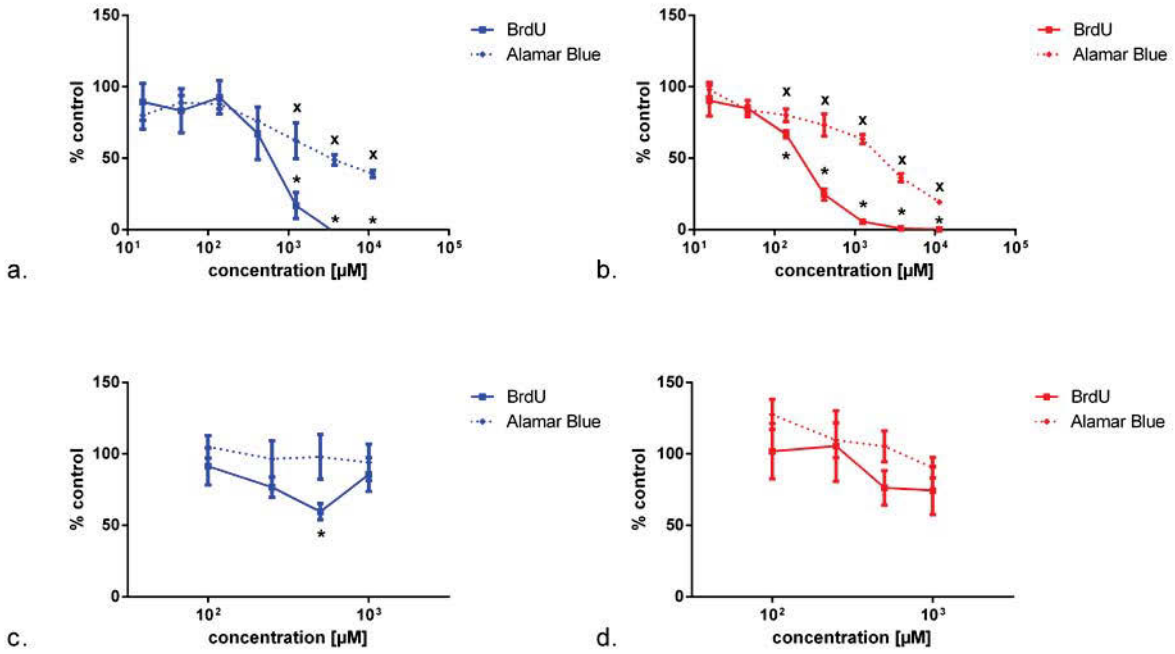
a.



b.

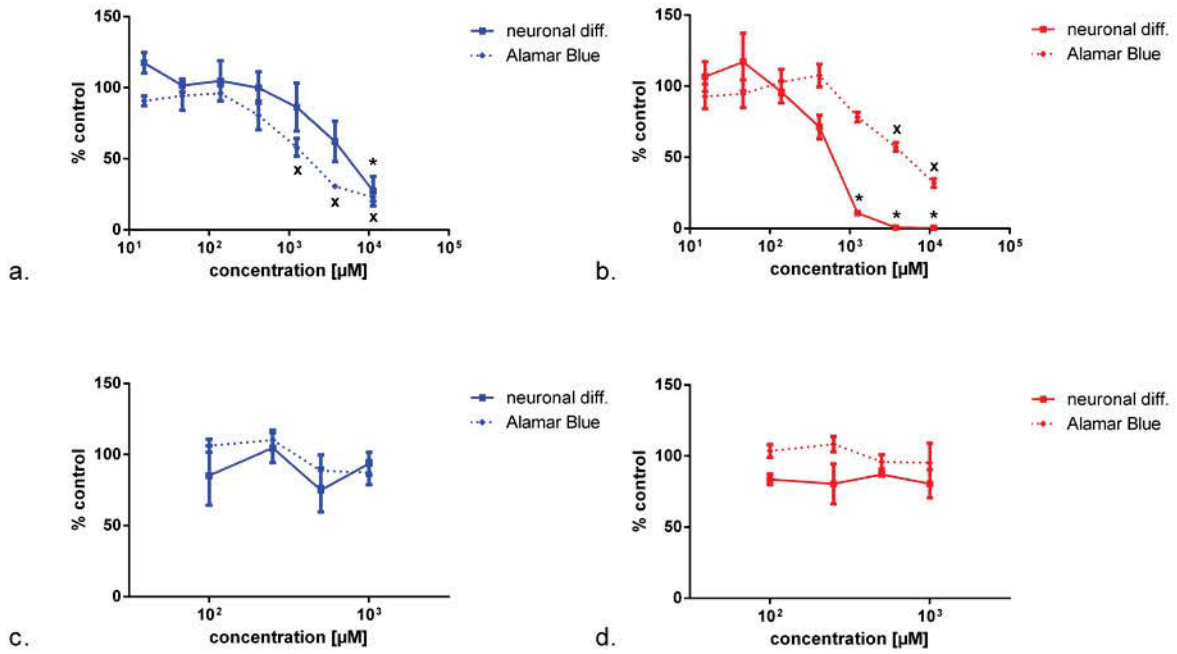
Supplementary Figure 4: Analysis of VPA metabolites in proliferating human and rat neurospheres. Proliferating human and rat neurospheres were treated with 1000 µM VPA for 48 hrs. At the end of exposure time, supernatant was collected and VPA metabolites were quantified using a gas chromatography – mass spectrometry (GC-MS) assay. **a.** Representative extracted ion chromatograms of rat neurospheres treated with 1000 µM VPA and respective standards are shown for ene-VPA metabolites and OH-VPA metabolites. For 4-keto-VPA chromatograms, see supplementary figure 3. **b.** Concentration of VPA metabolites, values are given in average nanomolar metabolite concentrations ± SD (n = 2-4 independent experiments).

Figure S5:



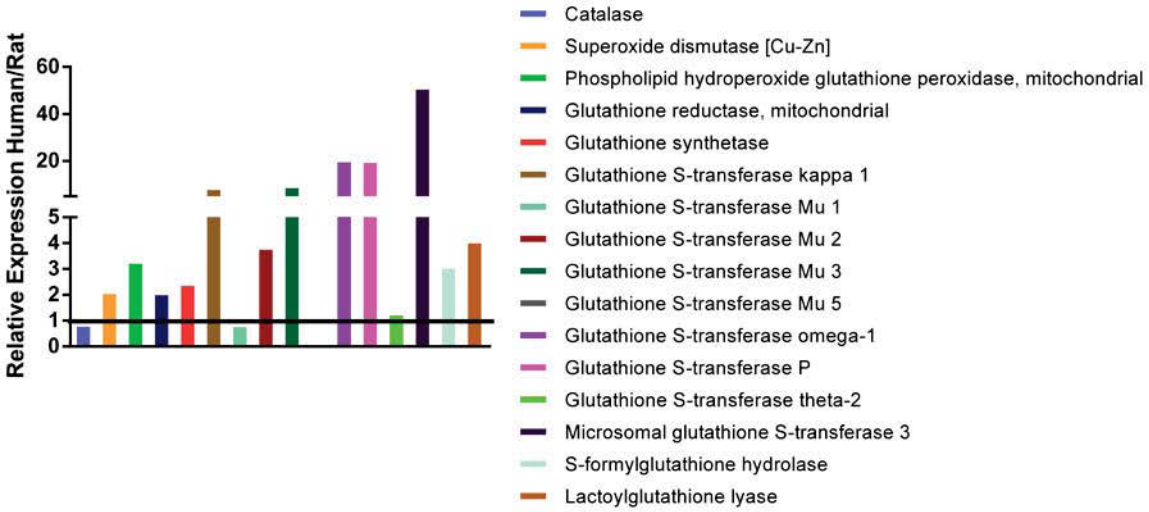
Supplementary Figure 5: Effects of sodium butyrate and valpromide on human and rat neurosphere proliferation. Proliferation and viability were assessed by testing 7 concentrations of sodium butyrate (a. and b.) and 4 concentrations of valpromide (c. and d.) in human (a. and c.) and rat (b. and d.) neurospheres. Therefore, 4 floating spheres per exposure group were plated one sphere per 96-well for 3 days. At the end of exposure time, proliferation was assessed by BrdU Assay and viability was monitored by Alamar Blue Assay. Concentration response curves are shown for human and rat neurospheres. Values are given as average percentages of solvent control for the endpoints proliferation (BrdU) and viability (Alamar Blue) ± SEM (n = 3 independent experiments). Asterisks (proliferation) and crosses (viability) denote significance respect to solvent control ($p < 0.05$).

Figure S6:



Supplementary Figure 6: Effects of sodium butyrate and valpromide on the amount of neurons in differentiating human and rat neurospheres. The differentiation of NPC to neurons and viability were assessed by testing 7 concentrations of sodium butyrate (a. and b.) and 4 concentrations of valpromide (c. and d.) in human (a. and c.) and rat (b. and d.) neurospheres. Therefore, 5 spheres per exposure group were plated in one well of a PDL/laminin coated 8-Well chamber slide for 3 days. At the end of exposure time, viability was monitored by Alamar Blue Assay and neurospheres were fixed, immunocytochemically stained for βIII-tubulin and counterstained with Hoechst. Immunofluorescent pictures were taken and neurons were quantified by manually counting. Concentration response curves are shown for human and rat neurospheres. Values are given as average percentages of solvent control for the endpoints neuronal differentiation (neuronal diff.) and viability (Alamar Blue) ± SEM (n = 3 independent experiments). Asterisks (neuronal differentiation) and crosses (viability) denote significance respect to solvent control ($p < 0.05$).

Figure S7:



Supplementary Figure 7: Antioxidant Defense Proteins in proliferating human and rat neurospheres. Protein expression of antioxidant proteins was determined by proteome analysis of proliferating human and rat neurospheres. Ratios of normalized abundances (n = 6 replicates) of human/rat neurospheres are given.

Supplementary Table 1: Primer Sequences of human and rat NPC

Gene	forward primer	reverse primer
β-actin Human	CAGGAAGTCCCTTGCCATCC	ACCAAAAGCCTTCATACATCTCA
β-actin Rat	CCTCTATGCCAACACAGT	AGCCACCAATCCACACAG
HDAC1 Human	GGCCATTTTCAAGCCGGTCA	TCCACACACTTGGCGTGTCC
HDAC1 Rat	GGCGAGCAAGATGGCGCAGA	GCGTTGGCTTTGTGAGGACGATAG
$p21^{Cip/WAF1}$ Human	GAGACTCTCAGGGTCGAAAA	CAACCGCCTAGTTTTTGTTT
$p21^{Cip/WAF1}$ Rat	GTGATATGTACCAGCCACAGG	GTGGCGAAGTCAAAGTTCCAC
Ascl1 Human	CGCCGGTCTCATCCTACTCG	TGCGATCACCCTGCTTCCAA
Ascl1 Rat	ATGGCGGGTTCTCCGGTTTC	TGCCATCCTGCTTCCAAAGTCC
ANT1 Human	CCTACCCCTTTGACACTGTT	CAATCTTCCTCCAGCAGTCAA
ANT1 Rat	TCCTATCCATTTGACACTGTCC	CAATCTTCCTCCAGCAGTCA

Supplementary Table 2: GO-Term Enrichment Analysis of human, rat and mouse NPC. a. Proliferating conditions, b. differentiating conditions

a.

Rat up vs human	Category	Term	Fold Enrichment	FDR
	Biological Process	GO:0006103~2-oxoglutarate metabolic process	30.606	2.217E-02
	Biological Process	GO:0006084~acetyl-CoA metabolic process	13.822	1.517E-02
	Biological Process	GO:0043648~dicarboxylic acid metabolic process	12.603	2.660E-02
	Biological Process	GO:0006732~coenzyme metabolic process	6.001	3.268E-04
	Biological Process	GO:0051186~cofactor metabolic process	4.709	6.122E-03
	Biological Process	GO:0055114~oxidation reduction	3.161	2.154E-05
	Molecular Function	GO:0051287~NAD or NADH binding	11.352	1.446E-03
	Molecular Function	GO:0050662~coenzyme binding	6.223	2.060E-06
	Molecular Function	GO:0048037~cofactor binding	5.952	6.300E-09
	Molecular Function	GO:0019842~vitamin binding	5.928	2.727E-03
	Molecular Function	GO:0000166~nucleotide binding	1.690	1.931E-02
Human up vs rat	**Category**	**Term**	**Fold Enrichment**	**FDR**
	Biological Process	GO:0009263~deoxyribonucleotide biosynthetic process	15.076	2.607E-02
	Biological Process	GO:0006695~cholesterol biosynthetic process	7.731	4.505E-03
	Biological Process	GO:0016126~sterol biosynthetic process	6.317	8.975E-03
	Biological Process	GO:0045454~cell redox homeostasis	4.786	3.347E-03
	Biological Process	GO:0051186~cofactor metabolic process	2.680	2.239E-02
	Biological Process	GO:0006260~DNA replication	2.645	4.264E-02
	Biological Process	GO:0055114~oxidation reduction	2.454	4.676E-10
	Molecular Function	GO:0016860~intramolecular oxidoreductase activity	5.643	2.334E-02
	Molecular Function	GO:0016667~oxidoreductase activity, acting on sulfur group of donors	5.498	2.996E-02
	Molecular Function	GO:0051015~actin filament binding	4.782	1.782E-02

	Molecular Function	GO:0050662~coenzyme binding	3.231	6.290E-05
	Molecular Function	GO:0048037~cofactor binding	3.053	2.030E-06
	Molecular Function	GO:0003779~actin binding	2.272	7.141E-03
	Molecular Function	GO:0008092~cytoskeletal protein binding	2.050	1.641E-03
	Molecular Function	GO:0003723~RNA binding	1.873	8.594E-04
	Molecular Function	GO:0000166~nucleotide binding	1.606	8.340E-09
	Molecular Function	GO:0017076~purine nucleotide binding	1.443	4.631E-03
	Molecular Function	GO:0032555~purine ribonucleotide binding	1.423	2.072E-02
	Molecular Function	GO:0032553~ribonucleotide binding	1.423	2.072E-02
Human up vs mouse	**Category**	**Term**	**Fold Enrichment**	**FDR**
	Biological Process	GO:0006695~cholesterol biosynthetic process	7.160	3.590E-02
	Biological Process	GO:0006260~DNA replication	2.939	2.637E-03
	Biological Process	GO:0034654~nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process	2.787	1.136E-02
	Biological Process	GO:0034404~nucleobase, nucleoside and nucleotide biosynthetic process	2.787	1.136E-02
	Biological Process	GO:0006412~translation	2.375	2.948E-03
	Biological Process	GO:0044271~nitrogen compound biosynthetic process	2.355	5.036E-03
	Biological Process	GO:0055114~oxidation reduction	2.234	9.227E-07
	Biological Process	GO:0006259~DNA metabolic process	1.921	4.630E-02
	Biological Process	GO:0006396~RNA processing	1.891	3.748E-02
	Molecular Function	GO:0004364~glutathione transferase activity	8.933	4.523E-03
	Molecular Function	GO:0016765~transferase activity, transferring alkyl or aryl (other than methyl) groups	5.267	6.166E-03
	Molecular Function	GO:0051015~actin filament binding	5.244	2.394E-03
	Molecular Function	GO:0003779~actin binding	2.253	1.190E-02
	Molecular Function	GO:0003723~RNA binding	2.074	3.442E-06
	Molecular Function	GO:0008092~cytoskeletal protein binding	2.009	4.919E-03
	Molecular Function	GO:0000166~nucleotide binding	1.494	2.694E-05

Mouse up vs human	Category	Term	Fold Enrichment	FDR
	Biological Process	GO:0006734~NADH metabolic process	34.510	1.128E-02
	Biological Process	GO:0006103~2-oxoglutarate metabolic process	27.608	3.290E-02
	Biological Process	GO:0046356~acetyl-CoA catabolic process	19.206	1.805E-04
	Biological Process	GO:0006099~tricarboxylic acid cycle	19.206	1.805E-04
	Biological Process	GO:0009109~coenzyme catabolic process	19.113	1.948E-05
	Biological Process	GO:0043648~dicarboxylic acid metabolic process	16.240	1.020E-05
	Biological Process	GO:0051187~cofactor catabolic process	16.031	9.099E-05
	Biological Process	GO:0006084~acetyl-CoA metabolic process	16.031	9.099E-05
	Biological Process	GO:0009060~aerobic respiration	12.621	4.115E-03
	Biological Process	GO:0045333~cellular respiration	9.677	2.742E-08
	Biological Process	GO:0022904~respiratory electron transport chain	8.628	3.344E-03
	Biological Process	GO:0022900~electron transport chain	8.234	3.396E-07
	Biological Process	GO:0015980~energy derivation by oxidation of organic compounds	6.902	1.448E-06
	Biological Process	GO:0006732~coenzyme metabolic process	6.857	4.820E-07
	Biological Process	GO:0006119~oxidative phosphorylation	6.761	2.439E-03
	Biological Process	GO:0006091~generation of precursor metabolites and energy	5.822	2.198E-12
	Biological Process	GO:0046034~ATP metabolic process	5.785	3.221E-02
	Biological Process	GO:0051186~cofactor metabolic process	5.380	2.428E-05
	Biological Process	GO:0009259~ribonucleotide metabolic process	4.883	2.333E-02
	Biological Process	GO:0055114~oxidation reduction	4.666	3.721E-18
	Biological Process	GO:0006631~fatty acid metabolic process	4.183	2.341E-02
	Biological Process	GO:0044271~nitrogen compound biosynthetic process	3.398	1.140E-02
	Molecular Function	GO:0051287~NAD or NADH binding	15.594	3.647E-09
	Molecular Function	GO:0051539~4 iron, 4 sulfur cluster binding	14.658	8.607E-03
	Molecular Function	GO:0051540~metal cluster binding	9.423	6.085E-03
	Molecular Function	GO:0051536~iron-sulfur cluster binding	9.423	6.085E-03

	Molecular Function	GO:0050662~coenzyme binding	8.388	8.836E-15
	Molecular Function	GO:0048037~cofactor binding	7.779	1.217E-18
	Molecular Function	GO:0050660~FAD binding	7.271	1.247E-02
	Molecular Function	GO:0009055~electron carrier activity	5.448	3.216E-07
	Molecular Function	GO:0019842~vitamin binding	5.235	1.006E-02
	Molecular Function	GO:0030554~adenyl nucleotide binding	1.959	6.364E-04
	Molecular Function	GO:0000166~nucleotide binding	1.935	1.053E-06
	Molecular Function	GO:0001883~purine nucleoside binding	1.929	1.053E-03
	Molecular Function	GO:0001882~nucleoside binding	1.916	1.321E-03
	Molecular Function	GO:0017076~purine nucleotide binding	1.747	8.779E-03
Mouse up vs rat	**Category**	**Term**	**Fold Enrichment**	**FDR**
	Biological Process	GO:0055114~oxidation reduction	3.231	1.158E-04
	Molecular Function	GO:0019842~vitamin binding	5.843	2.591E-02
	Molecular Function	GO:0048037~cofactor binding	5.547	4.542E-06
	Molecular Function	GO:0050662~coenzyme binding	4.960	1.722E-02
	Molecular Function	GO:0000166~nucleotide binding	1.876	7.332E-04
	Molecular Function	GO:0017076~purine nucleotide binding	1.800	4.221E-02

b.

Rat up vs human	**Category**	**Term**	**Fold Enrichment**	**FDR**
	Molecular Function	GO:0003779~actin binding	3.746	4.464E-03
	Molecular Function	GO:0008092~cytoskeletal protein binding	3.061	4.818E-03
Human up vs rat	**Category**	**Term**	**Fold Enrichment**	**FDR**
	Biological Process	GO:0006635~fatty acid beta-oxidation	9.275	1.971E-04
	Biological Process	GO:0009062~fatty acid catabolic process	7.214	2.810E-03

	Biological Process	GO:0022904~respiratory electron transport chain	7.009	1.488E-07
	Biological Process	GO:0019395~fatty acid oxidation	6.659	6.267E-03
	Biological Process	GO:0034440~lipid oxidation	6.659	6.267E-03
	Biological Process	GO:0042773~ATP synthesis coupled electron transport	6.324	9.925E-05
	Biological Process	GO:0042775~mitochondrial ATP synthesis coupled electron transport	6.324	9.925E-05
	Biological Process	GO:0006120~mitochondrial electron transport, NADH to ubiquinone	6.183	1.294E-02
	Biological Process	GO:0045333~cellular respiration	5.598	1.253E-07
	Biological Process	GO:0044242~cellular lipid catabolic process	5.592	2.311E-05
	Biological Process	GO:0046164~alcohol catabolic process	5.538	9.642E-06
	Biological Process	GO:0006096~glycolysis	5.526	3.761E-02
	Biological Process	GO:0046365~monosaccharide catabolic process	5.320	3.710E-04
	Biological Process	GO:0006007~glucose catabolic process	4.885	4.632E-02
	Biological Process	GO:0019320~hexose catabolic process	4.790	9.337E-03
	Biological Process	GO:0044275~cellular carbohydrate catabolic process	4.722	7.847E-04
	Biological Process	GO:0006119~oxidative phosphorylation	4.577	2.221E-04
	Biological Process	GO:0022900~electron transport chain	4.556	1.882E-05
	Biological Process	GO:0016052~carbohydrate catabolic process	4.332	2.371E-04
	Biological Process	GO:0016054~organic acid catabolic process	4.254	3.195E-04
	Biological Process	GO:0046395~carboxylic acid catabolic process	4.254	3.195E-04
	Biological Process	GO:0015980~energy derivation by oxidation of organic compounds	3.935	6.315E-05
	Biological Process	GO:0007005~mitochondrion organization	3.593	2.427E-03
	Biological Process	GO:0051186~cofactor metabolic process	3.511	2.355E-05
	Biological Process	GO:0006091~generation of precursor metabolites and energy	3.470	7.817E-10
	Biological Process	GO:0006732~coenzyme metabolic process	3.240	1.227E-02
	Biological Process	GO:0006979~response to oxidative stress	3.167	1.010E-02
	Biological Process	GO:0016042~lipid catabolic process	3.139	6.812E-03
	Biological Process	GO:0055114~oxidation reduction	3.067	7.756E-17
	Biological Process	GO:0005996~monosaccharide metabolic process	2.978	1.349E-03

	Biological Process	GO:0006631~fatty acid metabolic process	2.862	1.911E-02
	Biological Process	GO:0019318~hexose metabolic process	2.828	3.673E-02
	Biological Process	GO:0070271~protein complex biogenesis	2.104	7.467E-03
	Biological Process	GO:0006461~protein complex assembly	2.104	7.467E-03
	Biological Process	GO:0065003~macromolecular complex assembly	1.953	5.057E-03
	Biological Process	GO:0043933~macromolecular complex subunit organization	1.895	8.024E-03
	Molecular Function	GO:0051287~NAD or NADH binding	6.470	6.748E-04
	Molecular Function	GO:0003954~NADH dehydrogenase activity	5.984	1.552E-02
	Molecular Function	GO:0008137~NADH dehydrogenase (ubiquinone) activity	5.984	1.552E-02
	Molecular Function	GO:0050136~NADH dehydrogenase (quinone) activity	5.984	1.552E-02
	Molecular Function	GO:0016655~oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	5.729	8.068E-03
	Molecular Function	GO:0016209~antioxidant activity	5.475	3.594E-02
	Molecular Function	GO:0016651~oxidoreductase activity, acting on NADH or NADPH	4.791	5.551E-04
	Molecular Function	GO:0048037~cofactor binding	3.946	1.048E-10
	Molecular Function	GO:0050662~coenzyme binding	3.619	1.977E-05
Human up vs mouse	**Category**	**Term**	**Fold Enrichment**	**FDR**
	Biological Process	GO:0006635~fatty acid beta-oxidation	6.767	1.485E-02
	Biological Process	GO:0042775~mitochondrial ATP synthesis coupled electron transport	4.398	4.894E-02
	Biological Process	GO:0042773~ATP synthesis coupled electron transport	4.398	4.894E-02
	Biological Process	GO:0016054~organic acid catabolic process	3.755	4.992E-04
	Biological Process	GO:0046395~carboxylic acid catabolic process	3.755	4.992E-04
	Biological Process	GO:0016052~carbohydrate catabolic process	3.303	2.580E-02
	Biological Process	GO:0007005~mitochondrion organization	3.158	5.428E-03
	Biological Process	GO:0022900~electron transport chain	3.158	4.839E-02
	Biological Process	GO:0051186~cofactor metabolic process	3.012	2.032E-04
	Biological Process	GO:0055114~oxidation reduction	2.609	1.998E-13
	Biological Process	GO:0006091~generation of precursor metabolites and energy	2.300	6.107E-03

	Biological Process	GO:0006412~translation	2.290	3.606E-03
	Biological Process	GO:0043933~macromolecular complex subunit organization	1.841	1.933E-03
	Biological Process	GO:0065003~macromolecular complex assembly	1.823	6.770E-03
	Molecular Function	GO:0051287~NAD or NADH binding	5.189	6.921E-03
	Molecular Function	GO:0003697~single-stranded DNA binding	4.776	7.058E-03
	Molecular Function	GO:0016651~oxidoreductase activity, acting on NADH or NADPH	4.069	2.145E-03
	Molecular Function	GO:0043566~structure-specific DNA binding	3.105	3.684E-03
	Molecular Function	GO:0048037~cofactor binding	3.089	4.783E-07
	Molecular Function	GO:0050662~coenzyme binding	2.695	1.732E-02
	Molecular Function	GO:0003779~actin binding	2.360	1.043E-03
	Molecular Function	GO:0003723~RNA binding	2.169	4.242E-08
	Molecular Function	GO:0008092~cytoskeletal protein binding	2.010	2.322E-03
Mouse up vs human	**Category**	**Term**	**Fold Enrichment**	**FDR**
	Biological Process	GO:0009109~coenzyme catabolic process	20.325	1.048E-06
	Biological Process	GO:0006099~tricarboxylic acid cycle	18.380	2.510E-04
	Biological Process	GO:0046356~acetyl-CoA catabolic process	18.380	2.510E-04
	Biological Process	GO:0051187~cofactor catabolic process	17.046	6.230E-06
	Biological Process	GO:0006084~acetyl-CoA metabolic process	13.637	2.366E-03
	Biological Process	GO:0009060~aerobic respiration	13.588	3.681E-04
	Biological Process	GO:0045333~cellular respiration	7.627	5.426E-05
	Biological Process	GO:0015980~energy derivation by oxidation of organic compounds	5.505	9.474E-04
	Biological Process	GO:0006732~coenzyme metabolic process	4.835	1.093E-02
	Biological Process	GO:0051186~cofactor metabolic process	4.336	7.308E-03
	Biological Process	GO:0006091~generation of precursor metabolites and energy	3.883	1.993E-04
	Biological Process	GO:0055114~oxidation reduction	3.391	3.005E-08
	Molecular Function	GO:0050662~coenzyme binding	4.304	1.510E-02
	Molecular Function	GO:0048037~cofactor binding	4.171	4.763E-04

Rat up vs mouse	Category	Term	Fold Enrichment	FDR
	Biological Process	GO:0000375~RNA splicing, via transesterification reactions	4.697	1.114E-03
	Biological Process	GO:0000377~RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	4.697	1.114E-03
	Biological Process	GO:0000398~nuclear mRNA splicing, via spliceosome	4.697	1.114E-03
	Biological Process	GO:0008380~RNA splicing	3.275	6.560E-03
	Biological Process	GO:0006397~mRNA processing	3.029	1.320E-02
	Biological Process	GO:0016071~mRNA metabolic process	2.971	4.030E-03
	Biological Process	GO:0006396~RNA processing	2.473	9.730E-03
	Biological Process	GO:0045184~establishment of protein localization	2.199	8.683E-03
	Biological Process	GO:0008104~protein localization	2.157	3.096E-03
	Biological Process	GO:0015031~protein transport	2.108	4.079E-02
	Molecular Function	GO:0003779~actin binding	3.742	1.092E-05
	Molecular Function	GO:0008092~cytoskeletal protein binding	3.025	2.231E-05
Mouse up vs rat	**Category**	**Term**	**Fold Enrichment**	**FDR**
	Biological Process	GO:0009109~coenzyme catabolic process	15.767	7.800E-04
	Biological Process	GO:0051187~cofactor catabolic process	13.224	2.869E-03
	Biological Process	GO:0045333~cellular respiration	6.868	6.265E-04
	Biological Process	GO:0015980~energy derivation by oxidation of organic compounds	5.694	2.195E-04
	Biological Process	GO:0006732~coenzyme metabolic process	5.024	2.819E-03
	Biological Process	GO:0051186~cofactor metabolic process	4.467	2.197E-03
	Biological Process	GO:0006091~generation of precursor metabolites and energy	4.093	1.727E-05
	Biological Process	GO:0055114~oxidation reduction	3.288	7.925E-08
	Molecular Function	GO:0048037~cofactor binding	4.999	7.490E-07
	Molecular Function	GO:0016491~oxidoreductase activity	3.086	5.628E-07
	Molecular Function	GO:0003824~catalytic activity	1.586	2.214E-09

21st Century Risk Assessment: case study for species-specific hazard characterization of valproic acid

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3. Abschlussdiskussion

Die Identifizierung von Chemikalien mit entwicklungsneurotoxischem Potential gewinnt zunehmend wissenschaftlich sowie gesellschaftspolitisch an Bedeutung, da schon eine geringe Beeinflussung individueller kognitiver Fähigkeiten große Auswirkungen auf die gesellschaftliche Verteilung der Intelligenz nach sich zieht (Abbildung 1-1). So steigt die Inzidenz für Neuroentwicklungsstörungen wie Autismus, Aufmerksamkeitsdefizit-/Hyperaktivitätsstörungen (ADHS) und Legasthenie derzeit stetig (Grandjean and Landrigan 2014). Unter Berücksichtigung dessen sind sich Vertreter von wichtigen Interessengruppen (Regulatoren, akademische Wissenschaftler, Industrievertreter) einig, dass sicherheitstoxikologische Testungen auf Entwicklungsneurotoxizität für die Zulassung von Chemikalien essentiell sind (Bal-Price et al. 2015a). Von den tausenden von Chemikalien, die sich derzeit im Umlauf befinden, wurden jedoch bisher nur etwa 100 auf ihr entwicklungsneurotoxisches Potential in Richtlinienkonformen Tierversuchsstudien untersucht (Makris et al. 2009), was hauptsächlich durch den hohen Zeit- und Kostenaufwand sowie den enormen Tierverbrauch, welcher zur Durchführung der Richtlinienstudie benötigt wird, begründet ist (Crofton et al. 2012). Weiterhin bringen Speziesunterschiede das Problem der Extrapolation von Effekten im Versuchstier auf den Menschen mit sich (Leist and Hartung 2013). Dementsprechend sind Alternativen zu den verfügbaren regulatorische Testmetoden notwendig, um sowohl Zeit und Kosten zu reduzieren, als auch das Verständnis von Entwicklungsneurotoxizität und derer zu Grunde liegenden, bisher wenig verstandenen molekularen Mechanismen zu verbessern (Bal-Price et al. 2015a). Auch wenn *in vitro* Methoden die *in vivo* Versuche mittelfristig nicht vollständig ersetzen werden, so können Screeningassays trotzdem durch Priorisierung helfen, die Zahl der Tierversuche auf ein Minimum einzuschränken. Im Zusammenhang mit dem Paradigmenwechsel in der Toxikologie wird dabei die Verwendung von *in vitro* Modellen, die die menschliche Physiologie bestmöglich widerspiegeln, empfohlen (Gibb 2008).

In den letzten Jahren wurden verschiedene vielsprechende *in vitro* Modelle für das Screening auf Entwicklungsneurotoxizität entwickelt (Bal-Price et al. 2012). Eines dieser Modelle basiert auf normalen primären neuralen Progenitorzellen, die als Neurosphären kultiviert werden und in der vorliegenden Arbeit verwendet wurden.

Für die Festlegung von auf Tierversuchen basierenden regulatorischen Grenzwerten ist das Verständnis von Speziesunterschieden von großer Bedeutung. Daher ist es von Vorteil, wenn ein *in vitro* System die Möglichkeit bietet, mehrere Spezies komparativ nebeneinander zu analysieren. Gegenstand dieser Dissertation war es deshalb, einen auf Neurosphären basierenden *in vitro* Screeningassay für Entwicklungsneurotoxizitätstestung, der eine direkte Untersuchung von Speziesunterschieden zwischen Nager und Mensch zulässt, sowie des-

sen biologische Applikationsdomäne zu charakterisieren und molekulare Speziesunterschiede wie auch deren Bedeutung für den Prozess der Chemikalienrisikobewertung zu untersuchen.

3.1. Physiologische Relevanz des Neurosphärenmodells

Die Anwendung eines Zellmodells auf entwicklungsneurotoxikologische Fragestellungen setzt voraus, das es relevante Prozesse der Gehirnentwicklung abbilden kann. Bei der Verwendung von Primärzellen sollten diese außerdem ihre *in vivo* Eigenschaften während der *in vitro* Kultivierung weitestgehend behalten. Die in dieser Arbeit verwendeten humanen NPCs stammen aus dem zweiten Schwangerschaftstrimester des Menschen (Gestationswoche 16-19) und sollten daher Hirnentwicklungsprozesse der frühen fetalen Phase nachstellen können. Entsprechend wurden Nager NPCs aus der frühen postnatalen Phase präpariert (Clancy et al. 2007). In vergangenen Projekten der Arbeitsgruppe wurde bereits eine Reihe von molekularen Untersuchungen durchgeführt, um humane Neurosphären näher zu charakterisieren (Fritsche et al. 2011; Gassmann et al. 2010; Gassmann et al. 2014; Moors 2007; Moors et al. 2007; Moors et al. 2009; Schreiber et al. 2010). Für Nagerneurosphären fehlten solche Untersuchungen jedoch bisher weitestgehend, weshalb in der vorliegenden Arbeit vergleichende Untersuchungen in humanen und Nagerneurosphären hinsichtlich neuroentwicklungsrelevanter Prozesse durchgeführt wurden.

Neurosphären von Nager und Mensch erwiesen sich als geeignet für eine Untersuchung der Endpunkte Progenitorzell-Proliferation, Migration, Differenzierung in neurale Effektorzellen (Astrozyten, Neurone, Oligodendrozyten) und Apoptose. Diese Prozesse beginnen während der menschlichen Gehirnentwicklung in der späten embryonalen Phase und erstrecken sich über die fetale Phase, wohingegen sie in Nagern in die zweite Hälfte der Embyronalentwicklung sowie die frühe postnatale Entwicklung einzuordnen sind (Rice and Barone Jr 2000). Demensprechend behalten Neurosphären *in vitro* die entsprechenden Eigenschaften von Entwicklungsprozessen, die *in vivo* tatsächlich zu dem Zeitpunkt der Zellpräparation stattfinden. Dies spiegelt sich nicht nur in den basalen neuroentwicklungsrelevanten Eigenschaften der Neurosphären wider, sondern wurde in der Literatur bereits für eine Exposition von Nagern mit verschiedenen Noxen beschrieben. So induziert zum Beispiel das Antiepileptikum Valproinsäure (VPA) bei einer Exposition von schwangeren Ratten während der Embyronalphase (GD 12) die Progenitorzell-Proliferation sowie deren Differenzierung in Neurone sowohl *in vivo*, was sich in erhöhtem Hirngewicht und erhöhter Neuronendichte im Cortex äu-

ßerte, als auch *ex vivo* in Neurosphären, die aus den Progenitorzellen pränatal exponierter Rattennachkommen generiert wurden. Weiterhin konnten ähnliche Ergebnisse auch bei einer *in vitro* Behandlung mit VPA erzielt werden (Go et al. 2012). Dahingegen reduzierte eine postnatale Exposition von Mäusen mit VPA zwischen Postnataltag (PND) 7 und PND 14 *in vivo* die Proliferation der Progenitorzellen und Generierung junger, Doublecortin-positiver Neurone. Ähnlich zeigten Neurosphären, die *in vivo* oder *in vitro* mit VPA belastet wurden, ein gestörtes Proliferationsverhalten, welches sich in einer verminderten Sphärenbildung äußerte (Foti et al. 2013). Diese Ergebnisse sind im Einklang mit den Effekten, die ich in dieser Dissertation nach der Behandlung von Rattenneurosphären, präpariert aus PND 5 Rattenjungen, mit VPA detektiert habe (Publikation 2.4 und 2.5). So führte eine *in vitro* Behandlung mit VPA ebenso zu einer Inhibierung der Proliferation (EC_{50} = 380 µM) sowie der neuronalen Differenzierung (EC_{50} = 321 µM). Entsprechend scheinen also Neurosphären, die aus einem ähnlichen Stadium der Gehirnentwicklung generiert wurden, auch mit ähnlichen Effekten auf eine Substanzexposition zu reagieren, wodurch ihr Potential, Chemikalieneffekten in Abhängigkeit des jeweiligen Hirnentwicklungsstadiums untersuchen zu können, unterstrichen wird. Solche Studien verdeutlichen die physiologische Relevanz des Neurosphärenmodells für die *in vivo* Gehirnentwicklung und damit ihre Eignung für entwicklungsneurotoxikologische Fragestellungen.

Neben der Untersuchung der Abbildung von Hirnentwicklungsprozessen in Neurosphären auf rein funktionaler Ebene (Fähigkeit zur Proliferation, Migration und Differenzierung) ist es für eine nähere Charakterisierung hinsichtlich der physiologischen Relevanz des Zellsystems ebenso notwendig, die Präsenz und Funktionalität von Signalwegen, die während der Hirnentwicklung von Bedeutung sind, zu untersuchen. Zu diesem Zweck wurden in der vorliegenden Dissertation endpunktspezifische Kontrollen in humanen und Nagerneurosphären etabliert (Publikation 2.1 und 2.4). Dabei handelt es sich um Substanzen, die dafür bekannt sind, bestimmte Signalwege zu beeinträchtigen, die relevant für den jeweiligen Entwicklungsprozess sind (Crofton et al. 2011; Kadereit et al. 2012). Beispielsweise haben vergangene Arbeiten der Gruppe bereits gezeigt, dass die Proliferation EGF-abhängig ist und sich somit der Entzug dieses Wachstumsfaktors negativ auf die Proliferation der Neurosphären im Hinblick auf die Durchmesserzunahme auswirkt (Moors et al. 2009), was in der vorliegenden Arbeit für Nagerneurosphären bestätigt werden konnte. Zusätzlich wurde die Hemmung der Proliferation nicht nur indirekt durch eine verringerte Größenzunahme, sondern auch direkt durch eine Abnahme der DNS-Synthese, gemessen mittels Einbau des Thymidin Analogons Bromdesoxyuridin, nachgewiesen (Publikation 2.1 und 2.4). Solche Modelsubstanzen können nicht nur zur spezifischen Signalwegsanalyse eines Zellsystems eingesetzt werden,

sondern auch als Positivkontrollen in den Assays dienen (Crofton et al. 2011; Kadereit et al. 2012). Ein weiterer Ansatz, die physiologische Funktionalität des Neurosphärenmodells zu überprüfen, bietet eine Transkriptomanalyse von Neurosphären in verschiedenen Differenzierungsstadien und ein anschließender Vergleich mit *in vivo* Transkriptomanalysen vergleichbarer Hirnentwicklungsstadien von Mensch und Nager. Diese Arbeiten sind Gegenstand eines laufenden Validierungsprojektes, welches durch das Bundesministerium für Bildung und Forschung (BMBF) gefördert wird, und werden in Zukunft eine genaue Charakterisierung der biologischen Prozesse, die in den Neurosphären tatsächlich abgebildet werden können, ermöglichen, um so den biologischen Anwendungsbereich dieses Zellsystems zu definieren. Ein derartiger Ansatz ist für andere Zellsysteme, die für entwicklungsneurotoxikologische Untersuchungen eingesetzt werden, noch nicht publiziert, jedoch wurden Transkriptomanalysen in humanen embryonalen Stammzellsystemen, wenn auch ohne Bezug auf eine *in vivo* Relevanz der Genregulierungen, bereits angewendet, um den Einfluss der entwicklungsneurotoxischen Modellsubstanzen Methylquecksilber und VPA zu untersuchen (Krug et al. 2013).

Im Hinblick auf Speziesunterschiede zeigten humane und Nagerneurosphären basale Unterschiede sowohl auf molekularer wie auch auf funktioneller Ebene. So unterscheiden sie sich signifikant in ihrer proliferativen Kapazität wie auch in ihrer Fähigkeit, zu Neuronen und Astrozyten zu differenzieren, sowie in dem Reifegrad der differenzierten Zellen (Publikation 2.3). *In vivo* unterscheiden sich Mensch und Nager ebenfalls in ihrer Gehirnentwicklung hinsichtlich Morphologie und der Geschwindigkeit von Reifeprozessen (Semple et al. 2013). Beispielsweise weisen Primaten und Mäuse eine unterschiedliche Anzahl kortikaler Schichten sowie Aufteilung ihrer subventrikulären Zone auf, in der ein maßgeblicher Teil der Progenitorzellproliferation und Neurogenese stattfindet (Cheung et al. 2007). Weiterhin haben vergleichende Proteomanalysen in humanen und Nagerneurosphären deutliche Unterschiede in ihrer molekularen Ausstattung ergeben (Publikation 2.5). Sowohl proliferierende als auch differenzierte Neurosphären von Mensch, Maus und Ratte zeigten quantitative Unterschiede ihrer Proteomprofile in der Hauptkomponentenanalyse. Entsprechend zeigten Transkriptomanalysen der kortikalen Subplatte in Maus (GD 15,5-18,5) und Mensch (GW 15-21) differentielle Genexpressionsmuster (Miller et al. 2014), was die physiologische Relevanz molekularer Unterschiede während der Gehirnentwicklung von Mensch und Nager unterstreicht.

3.2. Der Neurosphärenassay als Screeningsystem für Entwicklungsneurotoxizität

Ein geeignetes *in vitro* Testsystem für die Evaluierung des entwicklungsneurotoxischen Potentials von Chemikalien muss zahlreichen Anforderungen gerecht werden, denn die Wahl des Testsystems kann das Testergebnis maßgeblich beeinflussen. So kann die Verwendung verschiedener Spezies (Mensch vs. Nager; Culbreth et al. 2012; Harrill et al. 2011) sowie verschiedener Zelltypen (Tumorzelle vs. Primärzelle; Radio et al. 2010) bei der Chemikalientestung zu unterschiedlichen Ergebnissen führen. Weiterhin weisen Fortschritte in Gewebetechnik und Biotechnologie darauf hin, dass Zellen, die als konventionelle 2D-Einzelzellschicht kultiviert werden, sich in ihrer Zellphysiologie deutlich von denen unterscheiden, die sich in einem dreidimensionalen Zellverband befinden (Alépée et al. 2014; Cukierman et al. 2001; Yamada and Cukierman 2007). Da das gewählte Zellsystem der (menschlichen) Physiologie möglichst nahe kommen sollte, wurden in der vorliegenden Arbeit humane und Nagerneurosphären als primäre 3D Ko-Kultur *in vitro* Systeme für die Entwicklungsneurotoxizitätstestung verwendet. Im vorherigen Abschnitt 3.1 wurde die physiologische Relevanz dieses Zellsystems bereits eingehend diskutiert.
Ein *in vitro* Screeningsystem für Entwicklungsneurotoxizität sollte u.a. (i) in der Lage sein, Schlüsselereignisse der Gehirnentwicklung abbilden zu können, (ii) eine Abgrenzung spezifischer neuroentwicklungsrelevanter Endpunkte von unspezifischen allgemein zytotoxischen Endpunkten zulassen, (iii) endpunktspezifische Kontrollen für die Überwachung der generellen Assay-Performance haben, (iv) die Möglichkeit eines erhöhten Durchsatzes bei der Chemikalientestung bieten und (v) hinsichtlich Sensitivität, Spezifität und Prädiktivität gut charakterisiert sein (Crofton et al. 2011). In den Publikationen 2.1 bis 2.4 dieser Dissertation wurde die Entwicklung des Neurosphärenassays als Screeningmodell für die Entwicklungsneurotoxizitätstestung unter Berücksichtigung dieser Kriterien ausführlich betrachtet. Die Abbildung von Schlüsselereignissen der Gehirnentwicklung sowie die Etablierung endpunktspezifischer Kontrollen wurde bereits im Abschnitt 3.1 diskutiert.

In Anbetracht der Tatsache, dass bisher nur ein sehr geringer Teil der sich im Umlauf befindlichen Chemikalien auf ihr entwicklungsneurotoxisches Potential getestet wurde (Makris et al. 2009), ist unter den oben aufgeführten Kriterien ein besonders wichtiges das Potential zur Durchsatzerhöhung, um eine schnelle und effiziente Testung einer Vielzahl von Chemikalien zu ermöglichen. Der Neurosphärenassay beinhaltet verschiedene Schritte, die für den Experimentator mit erhöhtem Zeitaufwand verbunden sind. So müssen z.B. Neurosphären eines homogenen Durchmessers sortiert und in 96-well Platten oder Objektträger ausplattiert wer-

den. In der vorliegenden Dissertation wurde deshalb die automatisierte Sortierung von Neurosphären mit Hilfe eines Durchflusszytometers für große Partikel (COPAS Large Particle Sorter) evaluiert, mit dem Ergebnis, dass keiner der zu testenden Endpunkte des Neurosphärenassays durch die automatisierte Zellsortierung nennenswert beeinflusst wird und sich die automatische Neurosphärensortierung damit für eine Erhöhung des Durchsatzes eignet (Publikation 2.2). Neben der Sortierung der Neurosphären sollten jedoch weitere Schritte des Neurosphärenassays für eine effizientere und zuverlässigere Chemikalientestung automatisiert werden. So können Chemikalien-Verdünnungsreihen für die Erstellung von Konzentrationswirkungskurven mit Hilfe eines Pipettier-Roboters hergestellt und aufwendige Messungen von Migrationsstrecken und Auszählungen von Zellkernen und Neuronen mit Hilfe eines automatischen Mikroskops durchgeführt werden. Letzteres ist Gegenstand laufender Arbeiten. Der Cellomics Arrayscan nimmt dabei immunzytochemische Färbungen differenzierter Neurosphären automatisch auf, und das in der Arbeitsgruppe entwickelte Programm “Omnisphero” evaluiert die Aufnahmen hinsichtlich Anteil, Verteilung und Morphologie differenzierter Zellen sowie Migrationsparametern wie Migrationsstrecke oder Anzahl migrierter Kerne (Schmuck et al. 2015). Eine automatische Bildauswertung findet bereits seit einigen Jahren vermehrt Anwendung in der Chemikalientestung auf Entwicklungsneurotoxizität (Harrill et al. 2011; Harrill et al. 2013; Radio et al. 2010).
Derartige Automatisierungsansätze helfen nicht nur dabei, den Probendurchsatz zu erhöhen, sondern minimieren auch die Experimentator-abhängigen Faktoren bei der Wahl der Neurosphären, der Auswertung von Migrationsstrecken und der Auszählung differenzierter Zellen sowie die Fehleranfälligkeit bei der Herstellung der Chemikalien-Verdünnungsreihen für den Neurosphärenassay.

Für das Screenen von Chemikalien auf ihr entwicklungsneurotoxisches Potential wurden in der vorliegenden Dissertation verschiedene Assays miteinander kombiniert, die eine Abgrenzung spezifischer entwicklungsneurotoxischer Effekte von der allgemeinen Zytotoxizität einer Chemikalie innerhalb des gleichen Versuchsansatzes zulassen (Neurosphärenassay). Um die Vorhersagekraft des Neurosphärenassays zu untersuchen, wurde ein „Trainingsset“ aus sechs Positiv- und drei Negativsubstanzen zur Ermittlung von Konzentrations-Wirkungsbeziehungen in humanen und Rattenneurosphären getestet. Die Ratte wurde hier als Nagerspezies der Maus vorgezogen, da in den Richtlinien für die Tierversuchsstudien vorwiegend die Ratte als Testspezies vorgeschlagen wird (OECD 2007; USEPA 1998). Ziel dieser Testung war es, (i) die Anwendbarkeit des Neurosphärenassays für die Testung einer moderaten Anzahl von Chemikalien zu überprüfen, (ii) historische Kontrolldaten zu generieren, um Validitätskriterien der verschiedenen Assays für spätere Testungen zu definieren

und (iii) eine Vorstellung über Vorhersageparameter wie die Sensitivität und Spezifität der Methode zu bekommen (Crofton et al. 2011). Die Ergebnisse dieser Chemikalientestung sind in Publikation 2.4 ausführlich dargestellt und diskutiert und haben ergeben, dass sich das Assaydesign auch bei der manuellen Durchführung aller praktischen und Evaluierungsschritte als geeignet für die Testung der neun Chemikalien in zwei Spezies erwiesen hat. Dabei konnten Konzentrationswirkungskurven über einen breiten Konzentrationsbereich für alle Endpunkte erstellt und sigmoidale Anpassungskurven berechnet werden. Um die Effekte der Chemikalien unter den verschiedenen Assays und den beiden Spezies miteinander vergleichen zu können, wurden in dieser Dissertation EC_{50} Werte berechnet. Dies ist die Konzentration, bei der eine Chemikalie eine 50 prozentige Änderung in einem Endpunkt hervorruft. Alternativ ist aber auch die Verwendung von EC_{30} Werten, No Observed Effect Concentrations (NOECs), Lowest Observed Effect Concentrations (LOECs) oder eines Vielfachen der Lösemittelkontroll-Standardabweichung denkbar (Bal-Price et al. 2012). Die Wahl dieser Kenngröße sollte dabei von der Variabilität der Lösungsmittelkontrollen abhängen und kann die Anzahl falsch positiver Antworten beeinflussen (Crofton et al. 2011). EC_{50} Werte und NOECs oder LOECs haben sich dabei in den letzten Jahren für toxikologische Screeningassays als gebräuchliche Effektgrößen etabliert (Culbreth et al. 2012; Harrill et al. 2011; Hayess et al. 2013; Stern et al. 2014).

Weiterhin ermöglichte die Chemikalientestung die Generierung historischer Kontrolldaten für humane und Rattenneurosphären, die in Publikation 2.1 näher beschrieben sind. Dabei ergab sich für jeden Endpunkt eine Spanne an Werten, innerhalb der sich sowohl die Lösemittelkontrollen als auch die endpunktspezifischen Kontrollen befinden müssen, damit der jeweilige Versuch als valide gilt und verlässliche Ergebnisse liefern kann. So ist zum einen sichergestellt, dass der jeweilige Endpunkt den zu untersuchenden Hirnentwicklungsprozess hinreichend gut abbildet, und zum anderem, dass der Endpunkt durch die Positivsubstanz erwartungsgemäß moduliert wird. Beispielsweise sollte sich der Anteil an Neuronen in humanen Neurosphären nach dreitägiger Differenzierung zwischen fünf und zehn Prozent bewegen, und durch EGF auf mindestens 30 Prozent des Lösemittelkontrollwertes reduziert werden. Ein geringerer Anteil als fünf Prozent Neurone in der Migrationsfläche führt hingegen erfahrungsgemäß zu großen Standardabweichungen in den Werten und kann damit eine höhere Anzahl an falsch positiven Effekten mit sich bringen.

Bei der Etablierung von Screeningassays für toxikologische Testungen ist die Bestimmung von Kenngrößen wie Sensitivität, Spezifität und Prädiktivität für die Vorhersagekraft des Assays durch die Testung einer angemessenen Anzahl von Referenzchemikalien essentiell (ICCVAM 1997). Die Liste an Referenzchemikalien sollte sowohl Positivsubstanzen, die *in vivo* nachgewiesenermaßen entwicklungsneurotoxisch sind, als auch Negativsubstanzen, die

in vivo keine Hinweise auf Entwicklungsneurotoxizität geben, enthalten. Die Sensitivität eines Assays ist definiert als der Anteil richtig erkannter Positivsubstanzen und die Spezifität gibt den Anteil richtig erkannter Negativsubstanzen an. Die Prädiktivität eines Assays gibt entsprechend die Häufigkeit richtiger Vorhersagen an (Crofton et al. 2011). Die Wahl der Referenzchemikalien ist dabei essentiell für die Berechnung dieser Kenngrößen. Die Liste an Chemikalien sollte eine große Bandbreite von Chemikalienklassen umfassen und die Chemikalien sollten hinsichtlich ihrer Toxizitätsprofile und zu Grunde liegenden Toxizitätsmechanismen gut charakterisiert und divers sein (Kadereit et al. 2012). Die Positivchemikalien, die in dieser Dissertation im Neurosphärenassay von Ratte und Mensch getestet wurden, umfassten als drei der nachgewiesenermaßen human entwicklungsneurotoxischen Umweltchemikalien Methylquecksilber, Natriumarsenit und Chlorpyrifos (Grandjean and Landrigan 2014), das entwicklungsneurotoxische Arzneimittel Natriumvalproat (Ornoy 2009), die *in vivo* Modellsubstanz Methylazoxymethanolazetat (Goldey et al. 1994) sowie das Organophosphat Parathion. Die Negativchemikalien hingegen umfassten das hepatotoxische Analgetikum Paracetamol, das Antibiotikum Penicillin G und den exzitatorischen Neurotransmitter Glutamat. Obwohl diese neun Chemikalien, wie in Publikation 2.4 diskutiert wurde, größtenteils unterschiedliche Wirkmechanismen aufweisen und zu unterschiedlichen Chemikalienklassen gehören, repräsentieren sie nur einen sehr kleinen Teil der Gesamtheit an Chemikalien. Deshalb ist für eine zuverlässige Bestimmung der Vorhersagkraft des Neurosphärenassays die Testung eines größeren Chemikalientestsets essentiell, weshalb sich im Rahmen eines vom BMBF geförderten Validierungsprojektes derzeit 30 weitere Chemikalien (20 Positivsubstanzen und 10 Negativsubstanzen) in der Testung befinden. Eine solche Testung wird mehr Gewissheit über die Vorhersagekraft und die biologische Applikationsdomäne des Neurosphärenassays für das Chemikalien-Screening geben.

Um festzulegen, wann ein Assay eine Positiv- oder Negativsubstanz als solche erkennt, wurden bisher verschiedene Ansätze gewählt, wie in Publikation 2.4 eingehend diskutiert wurde. Ein gängiges Kriterium liefert die Abgrenzung eines spezifischen Effektes von allgemeiner Zytotoxität (Harrill et al. 2011; Stern et al. 2014), aber auch die Berechnung mathematischer Prädiktionsmodelle hat im Bereich der *in vitro* Toxizitätstestung bereits Anwendung gefunden (Hayess et al. 2013; Scholz et al. 1999). Solche Ansätze basieren jedoch alleinig auf dem inhärenten Gefahrenpotential einer Chemikalie und beziehen tatsächliche Expositionslevel nicht mit ein. Aus diesem Grund wurde in der vorliegenden Dissertation ein Parallelogramm-Ansatz gewählt, um die Prädiktivität des Neurosphärenassays zu untersuchen (siehe Abbildung 1-7). Dabei wurden die EC_{50} Werte des jeweils sensitivsten Endpunktes im Neurosphärenassay für die einzelnen Spezies bestimmt, und, sofern verfügbar, mit tatsächlichen internen Expositionsleveln in Ratte und Mensch verglichen, um festzustellen,

ob die Konzentrationen, die *in vitro* einen Effekt erzielten, physiologisch relevant sind. Publikation 2.4 enthält eine umfassende Diskussion der *in vivo* Relevanz von den *in vitro* erzielten Ergebnissen für alle neun Chemikalien in humanen und Rattenneurosphären. Die Vergleiche der *in vitro* und *in vivo* Konzentration zeigten dabei eine korrekte Erkennung von vier der sechs Positivsubstanzen sowie aller drei Negativsubstanzen auf. Anhand dieses kleinen Chemikalien-Trainingssets errechnet sich die Sensitivität des Neurosphärenassays damit auf einen Wert von 67% und die Spezifität auf einen Wert von 100%. Entsprechend ergibt sich einer Prädiktivität von 78%.

Bei einem solchen Ansatz der Risikoabschätzung einer Chemikalie anhand von *in vitro* Ergebnissen darf jedoch nicht außer Acht gelassen werden, dass es sich bei den Effekt-Konzentrationen im Allgemeinen um die nominale Konzentration der Testchemikalie im Medium handelt. Diese kann sich allerdings maßgeblich von der intrazellulären Chemikalienkonzentration unterscheiden. So können sich Chemikalien wie Methylquecksilber in der Zelle anreichern und dort höhere Konzentrationen erreichen (Zimmer et al. 2011), oder aber schnell verflüchtigen oder zerfallen und damit zu geringeren intrazellulären Konzentrationen als ursprünglich im Medium eingesetzt führen (Kadereit et al. 2012; Smirnova et al. 2013). Auch eine Bindung der Chemikalien an das Versuchsgefäß oder Medienbestandteile wie Serumproteine und eine Metabolisierung der Chemikalie können die tatsächliche Chemikalienkonzentration beeinflussen (Adeleye et al. 2014; Scholz et al. 2013; van Thriel et al. 2012). Für eine zuverlässige Risikoabschätzung im Sinne des Parallelogrammansatzes sollten also idealerweise intrazelluläre Chemikalienkonzentrationen bekannt sein oder aber mithilfe kinetischer Modellierung abgeschätzt werden können (Croom et al. 2015; van Thriel et al. 2012).

Für eine regulatorische Akzeptanz von *in vitro* Alternativmethoden müssen diese in der Regel einen formalen Validierungsprozess durchlaufen, der verschiedene langwierige Schritte beinhaltet. Neben einer detaillierten Beschreibung des Testsystems, seiner Rationale und der Beziehung zwischen dem zu untersuchenden Endpunkt und dem zugehörigen biologischen Ereignis sowie der Testung einer Vielzahl von Referenzchemikalien muss auch die Intra- und Interlabor-Reproduzierbarkeit der Methode demonstriert werden (Hartung et al. 2004; OECD 2005). Aufgrund des hohen Zeit- und Kostenaufwandes, der mit einer solchen Validierung verbunden ist, besteht inzwischen immer mehr Konsensus darüber, dass dieser Prozess zweckmäßig anzupassen ist (Bal-Price et al. 2015a). Dabei soll die Art der Validierung dem regulatorischen Zweck, dem die Methode dienen soll, angepasst werden und so den Prozess einer regulatorische Akzeptanz vor allem durch den Wegfall der Interlaboratorien-Testungen beschleunigt werden (Judson et al. 2013). So wurde für Assays, die eine Priorisierung von Chemikalien für weiterführende, aufwendigere Testmethoden ermöglichen sol-

len, vorgeschlagen, anstelle einer formalen Validierung eine mechanistische Validierung durchzuführen, die eine Untersuchung der biologischen Relevanz von Toxizitätsmechanismen durch die Testung von im Menschen gut charakterisierten Chemikalien vorsieht (Hartung et al. 2013). Da jedoch die Anzahl an gut charakterisierten humanen Entwicklungsneurotoxinen sehr begrenzt ist (Grandjean and Landrigan 2014), kann in einem Ansatz wissenschaftlicher Validierung ein Parallelogrammansatz, wie er in dieser Arbeit verwendet wurde (siehe Abbildung 1-7), angewendet werden (Kienhuis et al. 2009), um die Validität und Relevanz einer Methode zu demonstrieren. Wie in den Publikationen 2.1 bis 2.4 ausführlich diskutiert wurde, hat sich der Neurosphärenassay von Ratte und Mensch unter Berücksichtigung der vorher genannten Kriterien als vielversprechend für eine valide *in vitro* Methode für die Entwicklungsneurotoxizitätstestung erwiesen, da eine physiologische Relevanz der Endpunkte sowie der Chemikalieneffekte aufgezeigt werden konnte.

3.3. Einbindung des Neurosphärenmodells von Ratte und Mensch in die moderne Chemikalienrisikoabschätzung für Entwicklungsneurotoxizität

Der Prozess der toxikologischen Risikobewertung von Chemikalien ist für die Gesellschaft von überragender Bedeutung. Er schützt spezifische Populationsgruppen wie Arbeiter, die in ihrem Arbeitsumfeld mit Chemikalien exponiert werden, Patienten, die pharmazeutisch behandelt werden sowie die gesamte Bevölkerung, die durch Nahrungsmittel, Konsumgüter und die Umwelt ständig gegenüber Chemikalien exponiert ist. Deshalb ist es entscheidend, dass die toxikologische Risikobewertung die besten gerade verfügbaren wissenschaftlichen Ansätze nutzt. Die gegenwärtig verwendeten Methoden der Risikobewertung sind jedoch seit Jahrzehnten unverändert. Der Standardansatz verwendet nach wie vor Hochdosis-Tierversuchsstudien zusammen mit humaner Expositionsabschätzung und konservativen Unsicherheitsfaktoren oder linearer Extrapolation, um zu bestimmen, ob eine spezifische Chemikalienexposition als „sicher“ oder „unsicher“ angesehen werden kann (Adeleye et al. 2014). Vor acht Jahren hat jedoch das US National Research Council in dem Bericht „Toxicity Testing in the 21st Century: A Vision and a Strategy“ einen Paradigmenwechsel in der toxikologischen Testung eingeleitet, weg von Hochdosis-Tierversuchsstudien hin zu *in vitro* Methoden, die in human relevanten Zell- oder Gewebsmodellen Änderungen in zellulären Signalwegen evaluieren (Collins et al. 2008; Krewski et al. 2010; NRC 2007).

Derzeit werden Entwicklungsneurotoxizitätsstudien von der Legislative für die Chemikalienzulassung nur sehr begrenzt gefordert, was mit den bezüglich Kosten und Tierverbrauch unzureichenden und wissenschaftlich strittigen Richtlinien für die Tierversuchsstudien zu-

sammenhängt (Smirnova et al. 2013). Kürzlich hat sich jedoch die Europäische Behörde für Lebensmittelsicherheit (European Food Safety Authority, EFSA) in einer wissenschaftlichen Stellungnahme des Ausschusses für Pflanzenschutz über die Entwicklungsneurotoxizität der beiden Pestizide Acetamiprid und Imidacloprid für eine verpflichtende Einreichung von Entwicklungsneurotoxizitätsstudien im Rahmen des EU-Zulassungsverfahrens ausgesprochen. Dabei wurde ganz spezifisch auf die Unsicherheiten, die mit den *in vivo* Studien in Verbindung stehen, hingewiesen und die Einbeziehung mechanistischer Informationen, die auf *in vitro* Daten basieren, dringend empfohlen (EFSA 2013). Die Notwendigkeit für die Testung auf Entwicklungsneurotoxizität unter Berücksichtigung modernerer Ansätze findet also inzwischen nicht nur in den USA, sondern immer mehr auch in Europa, sowohl in der wissenschaftlichen Gemeinschaft als auch auf regulatorischer Ebene, Zustimmung (Bal-Price et al. 2015a). In der vorliegenden Dissertation wurde deshalb untersucht, in wie fern die Ergebnisse der Chemikalientestung im Neurosphärenassay von Ratte und Mensch in solche moderneren Konzepte der Risikobewertung für Entwicklungsneurotoxizität eingebunden werden können (Publikationen 2.4 und 2.5).

Das neue Konzept zur Evaluierung des Gefahrenpotentials von Chemikalien für die Risikobewertung schließt eine computergestützte Analyse der Bioaktivitätsprofile von Chemikalien ein. Diese können mittels *in vitro* Assays beispielsweise durch die Messung von Biomarkern für spezifische Signalwege generiert werden. Daraus ergeben sich sogenannte „Chemikalien-Signaturen“ für verschiedene Endpunkte, die mit adversen Effekten *in vivo* im Rahmen des AOP-Konzeptes korreliert werden und Anwendung im Prozess von Chemikalien-Screening und Priorisierung für die Toxizitätstestung und Wirkstoffforschung finden können (Kleinstreuer et al. 2014; Knudsen et al. 2015; Villeneuve et al. 2014). Da bekannt ist, dass Signalwege sich sowohl qualitativ als auch quantitativ zwischen verschiedenen Spezies unterscheiden (Black et al. 2012; Gassmann et al. 2010; Thomas et al. 2012), ist in diesem Zusammenhang die Untersuchung spezies-spezifischer Chemikalien-Signaturen essentiell.
In der vorliegenden Arbeit wurde eine Fallstudie über die spezies-spezifischen Chemikalien-Signaturen von humanen und Rattenneurosphären für verschiedene funktionale Endpunkte unter Verwendung des human entwicklungsneurotoxischen Antiepileptikums VPA (Ornoy 2009) durchgeführt, wie in Publikation 2.5 ausführlich beschrieben ist. Humane und Rattenneurosphären unterschieden sich dabei nicht nur in ihrer Sensitivität gegenüber VPA in den Endpunkten Proliferation und Differenzierung zu Neuronen, sondern auch in dem Mechanismus, der dem Verlust junger Neurone zu Grunde liegt. Quantitative Analysen von Signalwegskomponenten in beiden Spezies haben dabei Expressionsunterschiede für die Histondeazetylase 1 (HDAC1) sowie den Adenin Nucleotid Translokator 1 (ANT1) auf mRNS und

Proteinebene ergeben, die auch *in vivo* in mRNS Proben von Hirnen entsprechender Entwicklungsstadien in Ratte und Mensch bestätigt werden konnten. Solche quantitativen Unterschiede in der molekularen Ausstattung können dazu führen, dass verschiedene Spezies unterschiedlich sensitiv auf eine Chemikalienexposition reagieren, wie es bereits anhand humaner und Mausneurosphären demonstriert wurde (Gassmann et al. 2010). Mit Hilfe solcher Signalwegsanalysen können also toxikodynamische Speziesunterschiede spezifischer Chemikalien untersucht werden, die für die Chemikalienrisikobewertung essentiell sind (siehe Abbildung 1-7). Ein ähnliches Konzept der Risikobewertung wurde erst kürzlich von der U.S. EPA beschrieben. Dabei werden humane und Rattendaten, *in vivo* wie auch *in silico* und *in vitro*, in einem modifizierten Parallelogrammansatz integriert, um Chemikalieneffekte auf molekularer Ebene vergleichen und so toxikodynamische Speziesunterschiede besser verstehen zu können. Eine Einbindung dieses Wissens in den Prozess der Risikobewertung kann so die Unsicherheit in der Extrapolation von Effekten in der Ratte auf den Menschen verringern und die Chemikalienrisikobewertung verbessern, indem die konservativen toxikodynamischen Unsicherheitsfaktoren des klassischen Ansatzes der Risikobewertung anhand der Informationen aus *in vitro* und *in silico* Interspeziesvergleichen angepasst werden (Burgess-Herbert and Euling 2013).

Das AOP-Konzept stellt nun einen Rahmen zur Verfügung, um solche Spezies-spezifischen Chemikalien-Signaturen mit tatsächlichen *in vivo* Effekten kausal in Verbindung zu bringen (Ankley et al. 2010; Kleinstreuer et al. 2014), wie es Abbildung 1-4 bereits beschrieben hat. VPA führt auf Populationsebene unter anderem zu einer erhöhten Inzidenz an Autismus in pränatal exponierten Kindern (Ornoy 2009). Die Endpunkte, die VPA in humanen und Rattenneurosphären moduliert hat, können entsprechend mit zellulären sowie funktionalen Schlüsselereignissen assoziiert werden. Die Verwendung von Neurosphären als 3D organoides Zellkultursystem für die Testung fungiert dabei als Brücke zwischen Effekten auf zellulärer Ebene und Effekten auf die Organfunktion und ermöglicht damit die Untersuchung des „Mode Of Action“ (MOA) einer Chemikalie. Die Anordnung jeder verfügbaren Information für eine Chemikalie und dessen Wirkung auf den verschiedenen Ebenen der biologischen Organisation in einem AOP kann die Kategorisierung von Chemikalien mit ähnlicher Struktur und biologischer Aktivität erleichtern. So können Chemikalien entsprechend ihrer initialen molekularen Ereignisse oder aber gemeinsamer Schlüsselereignisse gruppiert werden (Bal-Price et al. 2015a), um Vorhersagen für die adversen Effekte ungetesteter Chemikalien treffen zu können. In Bezug auf die Entwicklungsneurotoxizitätstestung im Neurosphärenassay bilden die *in vitro* Endpunkte Proliferation, Migration, Differenzierung und Apoptose funktionale Schlüsselereignisse der Hirnentwicklung, die direkt auf *in vivo* Endpunkte übertragen werden können und bei deren Störung ein adverser Effekt auf Verhaltensebene im Tier oder

Menschen zu erwarten ist. Chemikalien könnten also bezüglich ihres Potentials, den Endpunkt Proliferation oder Migration oder Differenzierung zu beeinflussen, gruppiert werden. Am Beispiel der VPA-Fallstudie zu dessen spezies-spezifischen molekularen Mechanismen kann außerdem eine Kategorisierung auf Basis zellulärer Schlüsselereignisse getroffen werden. So könnte eine Substanz, die ein HDAC Inhibitor ist, direkt mit einer Inhibierung der Progenitorzell-Proliferation in Ratte und Mensch in Verbindung gebracht werden. Und tatsächlich inhibieren auch andere HDAC Inhibitoren wie Natriumbutyrat, SAHA oder TSA *in vitro* die Proliferation von neuralen Progenitorzellen (Foti et al. 2013; Zhou et al. 2011). In humanen und Rattenneurosphären konnte dies mit dem HDAC Inhibitor Natriumbutyrat bestätigt werden (Publikation 2.5). Weiterhin wäre ausgehend von den Ergebnissen in dieser Dissertation zu erwarten, dass Ratte und Mensch sich in ihrer Sensitivität gegenüber HDAC Inhibitoren um einen Faktor von zwei unterscheiden. Dahingegen sollte eine Chemikalie, die reaktive Sauerstoffspezies generiert und so Caspase-abhängige Apoptose einleitet, wahrscheinlich nur in der Ratte, nicht aber im Menschen mit der apoptotischen Degeneration junger Neurone assoziiert werden (siehe Publikation 2.5).

Neben der Gruppierung von Chemikalien nach Struktur und Wirkmechanismen kann ein AOP ebenso dazu dienen, Teststrategien für die Testung auf Entwicklungsneurotoxizität zu entwickeln und *in vitro* Daten in solche Teststrategien zu integrieren (Bal-Price et al. 2015a). Wie bereits erwähnt stellen AOPs eine kausale Verbindung zwischen einem initialen molekularen Ereignis, zwischengeschalteten Schlüsselereignissen auf unterschiedlichen biologischen Organisationsebenen sowie einem adversen Ergebnis auf Populationsebene dar (Ankley et al. 2010; OECD 2013). *In vitro* Assays bieten dabei die Möglichkeit, Chemikalien hinsichtlich ihres Potentials, ein bestimmtes initiales molekulares Ereignis oder Schlüsselereignis auslösen zu können, zu untersuchen. Dafür muss jedoch das finale adverse Ereignis pathobiologisch gut untersucht und das initiale molekulare Ereignis bekannt sein, was für Neuroentwicklungsstörungen wie z.B. Autismus oder ADHS oft nicht gegeben ist und die Entwicklung von AOPs für Entwicklungsneurotoxizität erschwert (Bal-Price et al. 2015b). Aus diesem Grund wird der Fokus derzeit eher auf den mittleren Teil des AOPs gelegt, der deutlich besser erschlossen ist und Schlüsselprozesse der Hirnentwicklung wie Progenitorzell-Proliferation, Apoptose, Zellmigration und Differenzierung sowie Neuriten- und Dendritenwachstum, Myelinisierung, Synapsenbildung und neuronale Netzwerkbildung einschließt. AOPs können also dazu verwendet werden, Testmethoden zu sammeln, die den Effekt einer Chemikalie auf solche Schlüsselereignisse untersuchen können. Voraussetzung dafür ist jedoch, dass die Schlüsselereignisse, die diese Testmethoden untersuchen, auch tatsächlich prädiktiv für einen adversen Effekt sind, was allerdings für die oben genannten Hirnentwicklungsprozess gegeben ist (Bal-Price et al. 2015a). Für die Untersuchung dieser Prozesse auf

biochemischer oder funktionaler Ebene steht bereits eine Vielzahl von Testsystemen zu Verfügung, die zellbiologische und biochemische Endpunkte wie auch Transkriptom-, Epigenetik- oder Metabolomanalysen nutzen (Balmer and Leist 2014; Baumann et al. 2014; Harrill et al. 2011; Hayess et al. 2013; Hogberg et al. 2013; Moors et al. 2009; Theunissen et al. 2011; van Thriel et al. 2012). Unter der Annahme, dass die Testmethoden hinsichtlich ihrer biologischen Applikationsdomäne gut charakterisiert sind, lassen sie sich in den Kontext eines AOPs einordnen. Dadurch erhöht sich die generelle Verlässlichkeit von *in vitro* Ergebnissen, da die Daten auf Basis fundierten Wissens hinsichtlich Relevanz und Prädiktivität für einen adversen, neuroentwicklungsrelevanten Effekt erhoben wurden (Bal-Price et al. 2015a).
Aufgrund der Komplexität der Gehirnentwicklung wird ein einziges Zellmodell nicht in der Lage sein, eine korrekte Vorhersage für das entwicklungsneurotoxische Potential aller Chemikalien zu treffen. Vielmehr wird eine Kombination von Assays, die Schlüsselereignisse unterschiedlicher Entwicklungsstadien abgreifen, ein realistischeres Testungsszenario ermöglichen, wie es Abbildung 3-1 zeigt. Dabei gliedert sich der Neurosphärenassay in die frühe fetale Phase der Gehirnentwicklung ein.

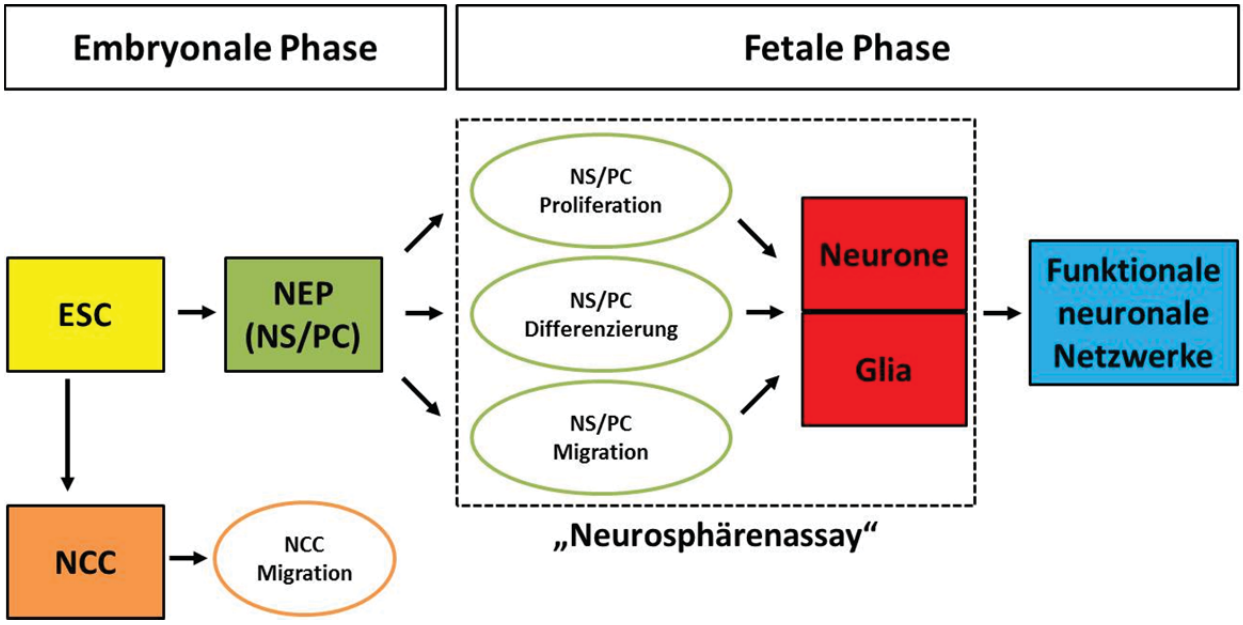

Abbildung 3-1: Teststrategie für die *in vitro* Entwicklungsneurotoxizitätstestung (modifiziert aus Publikation 2.4).
Die Untersuchung früher und späterer neuroentwicklungsrelevanter Schlüsselereignisse ermöglicht einen umfassenden Ansatz für die Entwicklungsneurotoxizitätstestung. Dabei ordnen sich die Endpunkte, die im Neurosphärenassay untersucht werden, in die frühe fetale Entwicklungsphase ein. ESC = Embryonale Stammzelle, NCC = Neuralleistenzelle, NEP = Neuroepithel-Vorläuferzelle, NS/PC = Neurale Stamm-/Progenitorzelle.

Die Idee, verschiedene Testmethoden miteinander zu kombinieren, um Daten für Entwicklungsneurotoxizität zu erfassen, wurde bereits vor einigen Jahren von der U.S. EPA aufgegriffen. So sollen sogenannte „Integrierte Evaluationsstrategien“ ein Rahmenwerk zur Verfügung stellen, um Daten in verschiedenen Ebenen der regulatorische Entscheidungsfindung zu generieren und zu verwenden (Bal-Price et al. 2012; Crofton et al. 2012). Diese Strategie beinhaltet vier verschiedene Informationsebenen, die schrittweise durchlaufen werden sollen: In Ebene 1 sollen die inhärenten Chemikalieneigenschaften untersucht und für QSAR (Quantitative Strukturaktivitätsbeziehung) und Analogieansatz („Read-Across“) basierte Evaluierungen verwendet werden. In Ebene 2 ordnen sich *in vitro* Ansätze, wie sie zuvor beschrieben wurden (Abbildung 3-1), ein. Das Ziel der Testungen in Ebene 1 und 2 ist eine Priorisierung von Chemikalien für weitere Testungen mit Hilfe von Screening Assays. In Ebene 3 wird auf eine Testung in komplexeren biologischen Systemen wie Modellorganismen oder komplexen *in vitro* Methoden zurückgegriffen und Ebene 4 sieht schließlich ein umfassendes Verständnis von Toxizitätsmechanismen unter Einbeziehung von Expositionsmodellierungen sowie eine Einordnung der Daten in AOPs vor (Abbildung 3-2).

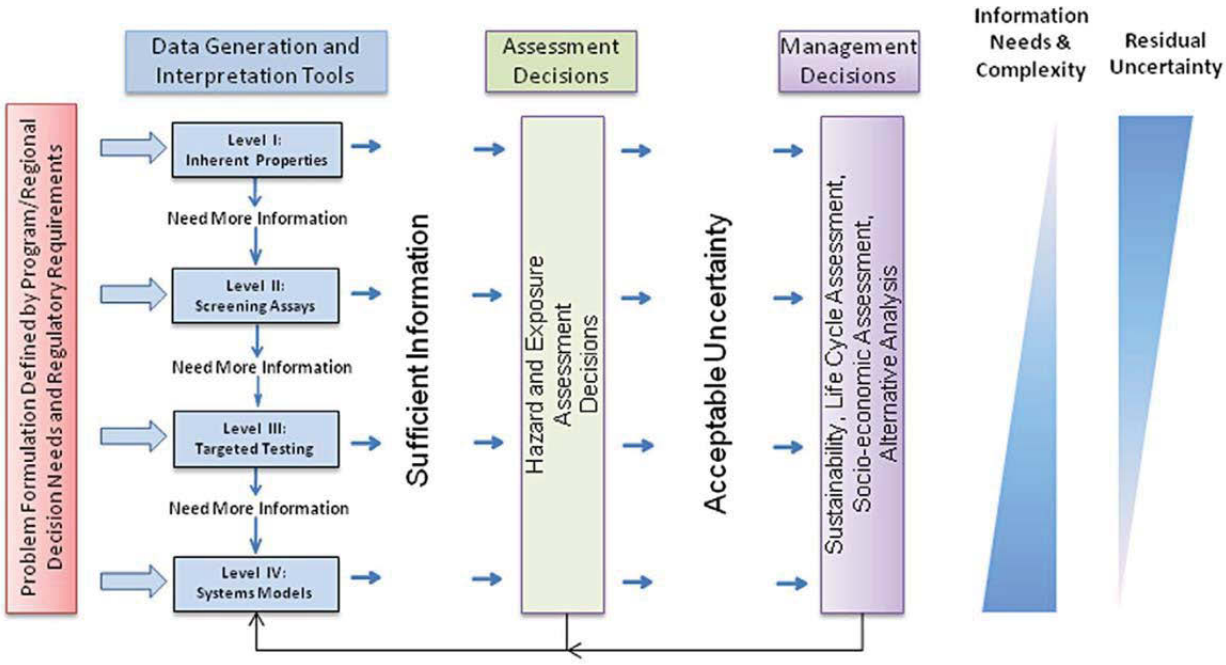

Abbildung 3-2: Integrierte Evaluationsstrategie für die Entwicklung neuer Testmethoden und Verwendung von Daten für Risikobewertungs- und Risikomanagement Entscheidungen (Crofton et al. 2012).
In dieser schrittweisen Evaluationsstrategie ist der Ressourcenverbrauch angepasst an die verschiedenen Ebenen der Datenerhebung, um schnell und effizient Daten generieren zu können. Für jede der vier Ebenen müssen neue Testmethoden entwickelt werden, die eine effiziente Untersuchung des Gefahrenpotentials von Chemikalien für das sich entwickelnde Nervensystem erlauben.

Ähnliche Ansätze werden im sogenannten „IATA“ (Integrated Approaches to Testing and Assessment) Konzept verfolgt, das inzwischen vermehrt in der regulatorischen Entscheidungsfindung angewendet wird (Patlewicz et al. 2015; Tollefsen et al. 2014). IATAs sind strukturierte Ansätze, die verschiedene Datentypen (*in vivo*, *in vitro*, *in silico, in chemico*) in die Identifizierung und Charakterisierung des Gefahrenpotentials einer Chemikalie oder einer Gruppe von Chemikalien einbeziehen. In einem IATA werden zunächst alle verfügbaren Informationen gesammelt und abgewogen. Falls die bereits existierenden Informationen für eine regulatorische Entscheidung nicht ausreichen, ist die Generierung neuer Daten in einem Hypothesen-gesteuerten Ansatz unter Einbeziehung der Informationen aus AOPs vorgesehen (Tollefsen et al. 2014).

Neben den neuen Konzepten für die Identifizierung und Charakterisierung des inhärenten Chemikalien-Gefahrenpotentials sind für die moderne Risikobewertung jedoch auch neue Ansätze für die Abschätzung der Exposition am Wirkungsort der Chemikalie im Organismus notwendig, denn vor allem für den Menschen sind interne Chemikalienkonzentrationen als Expositionsmaß rar. Die Vorhersage von Chemikalienkonzentrationen in humanen Körperflüssigkeiten oder Geweben ausgehend von der applizierten Dosis ist ein wichtiger Bestandteil in der Anwendung eines AOPs, da die Ergebnisse von *in vitro* Assays mit einer humanen Exposition in Verbindung gebracht werden müssen (Becker et al. 2015; Becker et al. 2014; Wetmore et al. 2011). Dabei sollten, wie bereits in Abschnitt 3.2 diskutiert wurde, genaue Chemikalienkonzentration in Medium und biologischer Matrix bekannt sein, um toxikokinetische Informationen über das Zellsystem zu erhalten. In einem Ansatz der „*in vitro in vivo* Extrapolation“ (IVIVE) werden dann *in vitro* Konzentrationen mit *in vivo* Konzentrationen verglichen. Da jedoch solche Expositionsdaten vor allem für den Menschen kaum zur Verfügung stehen, müssen diese mit Hilfe von PBPK- (physiologically based pharmacokinetic) Modellen abgeschätzt werden (Croom et al. 2015). Eine Berücksichtigung solcher Expositionsdaten wird schließlich die Interpretation der Relevanz von *in vitro* Ergebnissen, die im Rahmen eines AOPs erhoben wurden, erleichtern und so den Prozess der Chemikalienrisikobewertung verbessern (Patlewicz et al. 2015).

4. Zusammenfassung

Das Thema Entwicklungsneurotoxizität stößt sowohl wissenschaftlich als auch gesellschaftspolitisch seit einigen Jahren auf sehr großes Interesse, da schon eine geringe Beeinflussung der kognitiven Fähigkeiten große Auswirkungen auf die gesellschaftliche Verteilung der Intelligenz mit sich trägt. Die Prüfung von Chemikalien auf Entwicklungsneurotoxizität erfolgt derzeit noch im Tierversuch. Im Zuge des vom U.S. National Research Council eingeleiteten Paradigmenwechsels in der toxikologischen Testung wird jedoch die Entwicklung neuer Testmethoden und Modelle empfohlen, mit denen zum einen die Kosten und der zeitliche Aufwand einer Testung reduziert werden und zum anderen eine schnelle Datenerfassung zur Risikobewertung und eine bessere Identifizierung toxikologischer Mechanismen erfolgen kann. Zudem lässt die Extrapolation von im Tier erhobenen Befunden auf den Menschen und somit die Vorhersagbarkeit von Toxizitätsstudien im Tier wegen molekularer Speziesunterschiede zu wünschen übrig.

In der vorliegenden Dissertation wurde ein *in vitro* Screeningassay für die Entwicklungsneurotoxizitätstestung, der auf als Neurosphären wachsenden primären neuralen Progenitorzellen von Mensch und Ratte basiert, hinsichtlich Vorhersagekraft und biologischer Applikationsdomäne durch die Testung eines Chemikalien-Trainingssets charakterisiert. Weiterhin wurden anhand des Antiepileptikums Natriumvalproat molekulare Speziesunterschiede sowie deren Bedeutung für den Prozess der Risikobewertung von Chemikalien für die Gesundheit des Menschen genauer untersucht.

Die Ergebnisse dieser Arbeit demonstrieren die physiologische Relevanz des Neurosphärenmodells für die *in vivo* Gehirnentwicklung, denn humane und Rattenneurosphären bilden *in vitro* essentielle Hirnentwicklungsprozesse (Progenitorzell-Proliferation, Migration und Differenzierung zu neuralen Effektorzellen) ab, die *in vivo* zum Zeitpunkt der Zellpräparation tatsächlich ablaufen. Die Testung eines Trainingssets (Methylquecksilberchlorid, Natriumarsenit, Chlorpyrifos, Parathion, Methylazoxymethanolazetat, Natriumvalproat, Glutamat, Paracetamol und Penicillin G) im Neurosphärenassay von Ratte und Mensch, der Chemikalieneffekte auf die Endpunkte Proliferation, Migration und neuronale Differenzierung parallel zur Zellviabilität untersucht, ergab eine richtige Erkennung von vier der sechs entwicklungsneurotoxischen Positivsubstanzen und aller drei Negativsubstanzen unter Verwendung eines Parallelogrammansatzes. Die Sensitivität von humanen und Rattenneurosphären auf die Testchemikalien unterschied sich jedoch maßgeblich, wobei die Ratte in der Regel die empfindlichere Spezies war. Molekulare Untersuchungen zu der Spezies-spezifischen Entwicklungsneurotoxizität von Natriumvalproat zeigten, dass eine Inhibierung der Proliferation durch eine Histondeazetylase (HDAC) Inhibierung vermittelt wird und eine höhere Empfindlichkeit der Ratte wahrscheinlich auf eine höhere Expression von HDAC1 in Rattenneurosphären zurückzuführen ist. Im Gegensatz dazu ruft Natriumvalproat eine Verminderung des Anteils junger Neurone durch die Einleitung Caspase-abhängiger Apoptose nach oxidativem Stress in differenzierten Rattenneurosphären hervor, wohingegen es in humanen Neurosphären die neuronale Differenzierung ebenso durch eine HDAC Inhibierung hemmt. Unterschiede im Wirkmechanismus und auch die höhere Sensitivität der Ratte gegenüber Natriumvalproat scheinen durch eine vielfach höhere Expression des Adenin Nukleotid Translokators 1 (ANT1) begründet zu sein.

Die Ergebnisse der vorliegenden Dissertation zeigen, dass der Neurosphärenassay von Ratte und Mensch ein geeignetes *in vitro* System für die Entwicklungsneurotoxizitätstestung ist und die Möglichkeit bietet, molekulare Speziesunterschiede zu untersuchen. Die Einordnung solcher Ergebnisse in das „Adverse Outcome Pathway“ Konzept kann zu einem tieferen Verständnis entwicklungsneurotoxikologischer Speziesunterschiede beitragen und eine verbesserte Risikoabschätzung von Chemikalien für die menschliche Gesundheit ermöglichen.

5. Abstract

Developmental neurotoxicity is a huge social threat because small influences on individual cognitive abilities can have a big impact on the distribution of society's intelligence. Toxicological testing for developmental neurotoxicity is currently performed in animals. However, due to a paradigm shift in toxicology promoted by the U.S. National Research Council, the development of new test methods and models, which are less resource-intensive in terms of time and costs and enable a fast data generation and identification of toxicological mechanisms, is highly recommended. Moreover, due to species differences, the extrapolation of animal-derived findings and therefore the predictability of toxicological animal experiments for human hazards is insufficient.

In the present dissertation, an *in vitro* screening assay for developmental neurotoxicity testing, which is based on human and rat primary neural progenitor cells growing as neurospheres, was characterized regarding its predictive value and biological application domain by testing a training set of chemicals. Furthermore, molecular species differences in sensitivity towards the antiepileptic drug sodium valproate (VPA) were analyzed and placed into the context of chemical risk assessment for human health.

The results of this thesis demonstrate the physiological relevance of the neurosphere model for *in vivo* brain development as human and rat neurospheres mimic crucial neurodevelopmental processes (progenitor cell proliferation, migration, differentiation to neural effector cells) which actually take place *in vivo* at the time of cell preparation. Testing a training set of chemicals (methylmercury chloride, sodium arsenite, chlorpyrifos, parathion, methylazoxymethanol acetate, VPA, glutamate, acetaminophen and penicillin g) in the human and rat neurosphere assay, which analyses chemical effects on the endpoints proliferation, migration and neuronal differentiation in parallel to cell viability, revealed a correct classification of four out of six developmental neurotoxic positive compounds and all three negative compounds by applying a parallelogram approach. However, human and rat neurospheres differed in their sensitivity towards the testing chemicals with the rat being in general the more susceptible species. Molecular investigations of the species-specific developmental neurotoxicity of VPA showed that an inhibition of proliferation is mediated by histone deacetylase (HDAC) inhibition and a higher sensitivity of the rat is probably attributed to a higher HDAC1 expression in rat neurospheres. In contrast, VPA reduced the amount of young neurons in differentiated rat neurospheres by inducing caspase-dependent apoptosis which is due to oxidative stress, whereas HDAC inhibition seems to be the responsible mechanism for an inhibition of neuronal differentiation in human neurospheres. Divergent toxicity mechanisms and the higher sensitivity of rat neurospheres to sodium valproate seem to be connected to a substantially higher expression of adenine nucleotide translocator 1 (ANT1) in rat neurospheres.

The results of this thesis show that the human and rat neurosphere assay is a suitable *in vitro* method for developmental neurotoxicity screening of chemicals and enables an investigation of molecular species differences. The integration of such results into the "adverse outcome pathway" concept may contribute to a deeper understanding of species differences in developmental neurotoxicity and thus enable an improved chemical risk assessment for human health.

Abkürzungsverzeichnis

2D	zweidimensional
3D	dreidimensional
ADHS	Aufmerksamkeitsdefizit-/Hyperaktivitätsstörung
ANT	Adenin Nucleotide Translokator
AOP	Adverse Outcome Pathway
BMBF	Bundesministerium für Bildung und Forschung
BrdU	Bromdesoxyuridine
COPAS	Complex Object Parametric Analyzer and Sorter
DNS	Desoxyribonukleinsäure
DNT	Developmental Neurotoxicity
EC	Effektive Konzentration
EFSA	European Food Safety Authority
EGF	Epidermal Growth Factor
ESC	Embryonale Stammzelle
EU	Europäische Union
EZM	Extrazellulärmatrix
FGF	Fibroblast Growth Factor
GD	Gestationstag
GFAP	saures Gliafaserprotein
GW	Gestationswoche
HDAC	Histondeazetylase
IATA	Integrated Approaches to Testing and Assessment
ICC	Immunzytochemie
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IQ	Intelligenzquotient
ISTNET	International Stakeholder Network
IVIVE	*in vitro in vivo* Extrapolation
LOEC	Lowest Observed Effect Concentration
MOA	Mode of Action
NCC	Neuralleistenzelle
NEP	Neuroepithel-Vorläuferzelle
NOEC	No Observed Effect Concentration
NPC	Neurale Progenitorzelle

NRC	National Research Council
NS/PC	Neurale Stamm-/Progenitorzelle
OECD	Organization for economic cooperation and development
OPPTS	Office of prevention, pesticides and toxic substances
PND	Postnataltag
QSAR	Quantitative Strukturwirkungsbeziehung
REACH	Registrierung, Evaluierung, Autorisierung und Restriktion von Chemikalien
TUBB-3	βIII-Tubulin
USA	United States of America
U.S. EPA	United States Environmental Protection Agency
VPA	Valproinsäure

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Eidesstattliche Erklärung

Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder einer ähnlichen Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Jenny Baumann

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