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Neurotoxins

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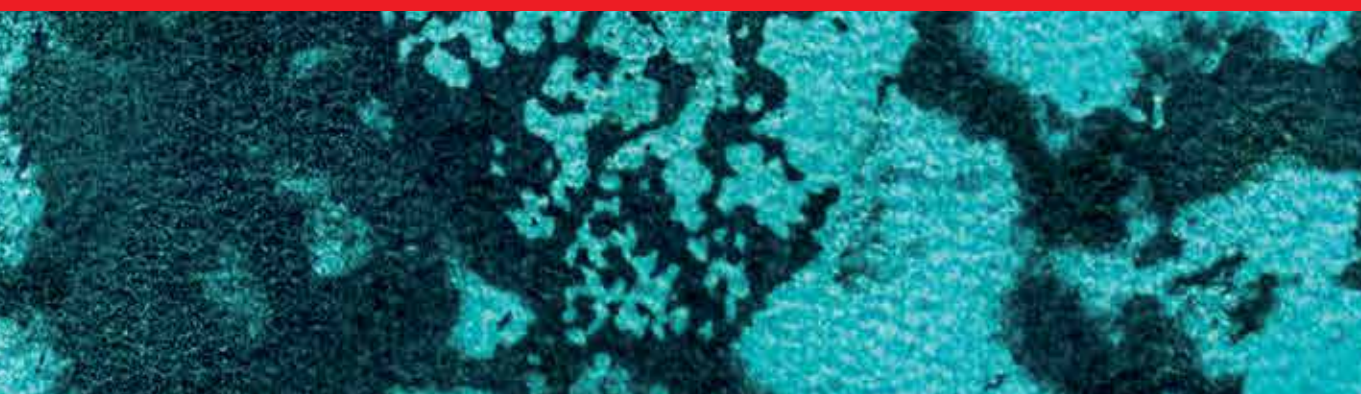

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Contributors

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Notice

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Meet the editor

J. Eric McDuffie, BSc, MBA, PhD, is the scientific director at Janssen Research and Development. He joined Janssen in 2007 and currently serves as the head of the Mechanistic and Investigative Toxicology in San Diego, California, USA. Previously, he held various positions at Pfizer, Inc. in Ann Arbor, Michigan, USA, including manager/senior scientist, Global Laboratory Core (2000–2001), and manager/principal scientist, Investigative Pathology (2001–2005). He also served as the principal scientist, Investigative Immunotoxicology at Esperion Therapeutics, a Pfizer, Inc. company, Plymouth, Michigan, USA (2006–2007). As a postdoctoral research fellow at the University of Michigan Medical School in Ann Arbor, Michigan, USA (1998–2000), he collaboratively investigated the role of pro- and anti-inflammatory cyto- and chemokines in liver, kidney, heart, and lung injury responses. He has coauthored several peer-reviewed manuscripts and book chapters and served as a coeditor for the benchmark book, *Drug Discovery Toxicology: From Target Assessment to Translational Biomarkers* (2016). His plethora of research interests spans across investigative toxicologic pathology in drug development. Current investigative foci include the assessment of excitotoxicity risk using novel human iPSC-derived glutamatergic neuronal models.

Contents

Preface

The book *Neurotoxins* was envisaged to serve as a "one-stop reference" for selected current topics, including translational cell models, various compounds and related neurotoxic and/or therapeutic effects, biomarkers, and advanced decision trees for diagnosing neurotoxicity. The book is organized into eight chapters: (1) "Using Human Pluripotent Stem Cell-Derived Neural Cultures to Assess Safety of New Drugs and Chemicals," (2) "Induced Pluripotent Stem Cell-Derived Human Glutamatergic Neurons as a Platform for Mechanistic Assessment of Inducible Excitotoxicity in Drug Discovery," (3) "Use of the Model Organism *Caenorhabditis elegans* to Elucidate Neurotoxic and Behavioral Effects of Commercial Fungicides," (4) "Neurotoxins and Autism," (5) "Botulinum Neurotoxin: A Multifunctional Protein for the Development of New Therapeutics," (6) "Resistance to Botulinum Toxins in Aesthetics," (7) "Developmental Neurotoxicity of Fluoride: A Quantitative Risk Analysis toward Establishing a Safe Dose in Children," and (8) "Targeting Acid Sensing Ion Channels by Peptide Toxins".

A significant challenge for toxicologists is understanding the unique signaling mechanisms responsible for neurotoxins. To address this issue, tactical approaches including but not limited to high-throughput assays as well as epidemiological data have been emphasized in the context of inducible neurotoxicity, which may progress silently in both humans and animals, before remarkable late-stage signs are evident. Hence, the needs and utilities for more predictive translational models to enable mechanistic investigations have been concisely described in this book.

This book was developed to serve as an open-access resource to academic, pharmaceutical, and environmental scientists, as well as students. Thus, I am sincerely grateful to the authors and peer reviewers for their contributions that helped to make this a benchmark publication. The astute assistance, guidance, and dedication of the *IntechOpen* staff are appreciatively acknowledged.

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Chapter 1

Using Human Pluripotent Stem Cell-Derived Neural Cultures to Assess Safety of New Drugs and Chemicals

Cassiano Carromeu

Additional information is available at the end of the chapter

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Abstract

The central nervous system (CNS) is a central pillar in safety pharmacology studies of new drugs. Characterization of serious adverse drug reactions to a new chemical entity involves extensive investigation using *in vitro* and *in vivo* models. However, primary culture of human neurons *in vitro* can be challenging, giving limited sample availability. Additionally, the inter-species differences between humans and current animal models impose a considerable obstacle to successfully predict the outcome of new drugs. New technologies also need to help address the 3Rs principles in animal research. Human pluripotent stem cells (hPSC) have the potential to change the current paradigm in pharmacological research. By using hPSCs and state-of-the-art differentiation protocols, researchers now have available an unlimited source of neural cells, able to mimic early and late stage of human CNS development. Moreover, hPSC-derived cells can be used at early stages of drug development, improving clinical predictability and reducing overall drug development costs. This chapter covers the advancements that resulted in hPSC-derived models intended to enable neurotoxicity assessment and drug screening. Finally, this chapter will also reveal the bottlenecks and the challenges to overcome of using hPSC as a predictive tool in research.

Keywords: human pluripotent stem cells (hPSC), induced pluripotent stem cells (iPSC), multielectrode array (MEA), Zika virus, neurotoxicity

1. Introduction

1.1. New therapeutic compounds and the nervous system

The human central nervous system (CNS) is a unique structure organized in an intricate network composed of different cell types [1, 2]. Its homeostasis is maintained by an orchestrated

signaling milieu composed of neurotransmitters, cell-cell interactions, and protein factors. Any compound acting upon one of the CNS components could potentially shift this delicate balance, resulting in untoward outcomes. Therefore, safety pharmacology profiling for compounds that crosses the blood-brain barrier represents a key step in the drug development process, particular prior to conducting studies in human subjects. Recently, the Biotechnology Innovation Organization (BIO) released the largest study of clinical drug development success rates to date [3]. In partnership with Amplion and Biomedtracker, BIO collected and analyzed a total of 9985 phase transitions in clinical trials between 2006 and 2015. Their data revealed a likelihood of approval being of only 9.6% for all developmental candidates. If segmented by diseases, candidates to neurology and psychiatry disorders fall under 9.6%, with 8.4 and 6.2% likelihood of approval, respectively. Moreover, adverse effects to the CNS account for a considerable proportion of all drug attrition cases. This demonstrates the poor predictability of current animal and *in vitro* models leveraged at the pre-clinical drug development stage.

Assessing the toxicological profile of new molecular entities requires extensive investigation using *in vitro* and *in vivo* models (**Figure 1**). This incremental accumulation of data helps to evaluate the toxicological profile and potential side effects of new compounds before moving to clinical trials. Studies to investigate the toxicity of drugs on the human central nervous system (CNS) relies mostly on animal (*in vivo*) and cellular (*in vitro*) models [4]. Although significant achievements have been accomplished using these models, there are many bottlenecks to overcome. For instance, efforts to fully recapitulate the human nervous system using animal models can be very challenging [5]. Rodents and human brains display major genetic, cellular, and anatomical differences [6]. Many compounds have failed in clinical trials even after being considered promising based on rigorous testing in animal models. Therapies to Alzheimer's disease (AD) for example have an attrition rate of 99.6% [7]. Many potential therapeutic compounds

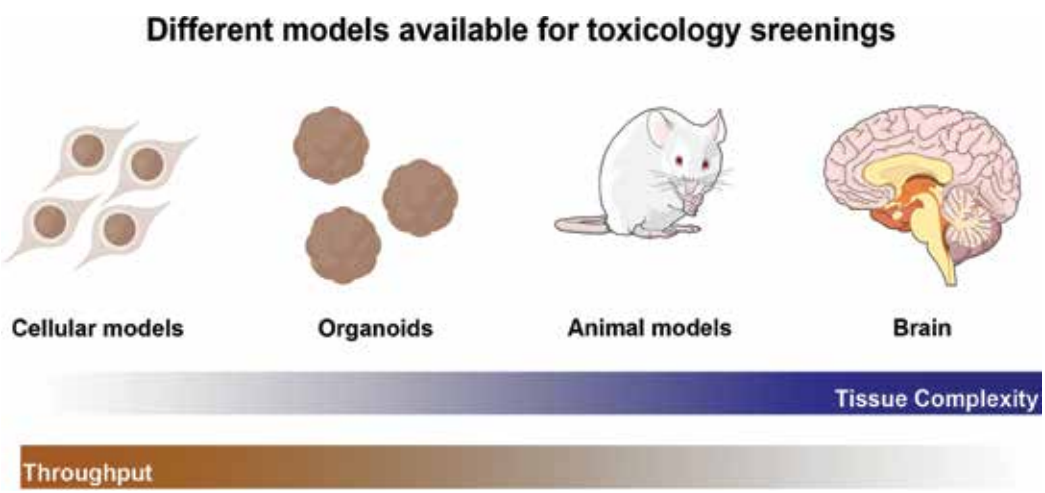

Figure 1. *In vitro* and *in vivo* models available for toxicological screenings. As the complexity of the model increases, there is a substantial decrease in throughput. Common cellular models include immortalized cell lines, primary tissue culture and hPSC-derived cells. Organoids are 3D structures derived from hPSC differentiation toward neuroectoderm in suspension.

for AD displayed unacceptable toxicity in humans. Additionally, while animal models have unquestionable importance in toxicological studies, new technologies could uniquely help to address the 3R principles of refine, reduce, and replace their use in this research space [8].

There are two main *in vitro* cellular models available for toxicology studies: primary and established cell lines [9, 10]. Primary cell lines are isolated directly from tissues. Their main advantage is that they more closely remember the *in vivo* counterpart, displaying many features presented in the target tissue. However, primary cell lines need fresh tissue to establish the cell culture and have limited capability of expansion *in vitro*. This turns impractical Studies that require large numbers of cells, especially from difficult-to-obtain tissue such as the human central nervous system. Immortalized cell lines, on the other hand, can be kept in culture for extensive periods of time and expanded through passaging. The immortalized cells have the intrinsic ability to proliferate indefinitely in culture, usually acquired by multiple mutations or transformations in their genomes. Although the proliferative potentials for immortalized cells make them amenable to large-scale production, they may significantly differ from the tissue of origin. Given the limitations imposed by these types of cell culture, human pluripotent stem cells have gained credence as a new reliable source of human tissues, with many advantages over the traditional *in vitro* cellular models.

2. Human pluripotent stem cells (IPSC)

Human pluripotent stem cells (hPSC) have the ability to expand to large amounts and differentiate into any cellular tissue of the body [11]. Giving these extraordinary abilities, hPSC can potentially change the current paradigm in pharmacological research, offering unlimited access to a reliable source of neural tissue able to mimic early and late stages of human CNS development [2]. There are two types of hPSC: embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC). Although both types share the same core features that classified them as hPSC, such as the ability of differentiating to any adult tissue, there are major differences between them. The hESC are derived from inner cell mass of blastocyst stage embryos after 5 days from the fertilization of the oocyte [12]. As of January 2017, 378 hESC lines were eligible for NIH fund research [13]. Comparatively, this library is still small to fully explore the whole human genomic diversity landscape. Moreover, giving its origin, hESC carry many ethical issues [14]. In spite of that, hESC have been pivotal on advancing the human stem cells research, permitting unlimited access to any human tissue of interest for the first time.

In 2006, a scientific breakthrough introduced a technique able to generate pluripotent stem cells without the ethical controversies of embryonic stem cells [15]. The team used the technique of reprogramming to reverse an adult mouse cell (fibroblast) into a pluripotent stem stage: the induced pluripotent stem cells (iPSC). Soon after the same research group published the technique using human fibroblast to generate human-induced pluripotent stem cells (hiPSC) [16]. Once hiPSC have the same capacity as their hESC counterparts to generate human target cells *in vitro*, many scientists have shifted their focus into producing patient-specific iPSC to potentially validate disease phenotypes *in vitro* [17]. For the nervous system, many studies confirmed the great potential of hiPSC in recapitulating CNS diseases [18, 19].

Moreover, studies have revealed the potential of using hiPSC as a drug-screening platform to systematically evaluate spontaneous neurological disorders and drug-induced neurotoxicity [17]. The biggest challenge for this approach is to identify key phenotypes *in vitro* for reproducible outcomes. Neurodevelopmental disorders, for example, impose such a significant challenge. Recently, two studies that focused on different diseases (Rett Syndrome and MeCP2 Duplication Syndrome) successfully demonstrated the use of hiPSC-derived cells in identifying potential therapeutic candidates [20, 21]. Both neurodevelopmental disorders altered MeCP2 gene expression (loss of a functional copy in Rett Syndrome and overexpression in MeCP2 Duplication Syndrome). These studies identified core alterations in the synapses of neurons in both conditions. In the MeCP2 Duplication Syndrome study, researchers developed a simplified drug-screening platform able to quickly assess the synaptic phenotype. By using a library of epigenetic modifiers, they identified two compounds that able to reverse the synaptic phenotype *in vitro*. However, the study also displays an alarming finding: although both potential therapeutic compounds identified in the study induced rescue of the cellular synaptic phenotype *in vitro*, one of them demonstrated significant toxicity on the CNS function in selected electrophysiology assays. This study highlights the need for an extensive characterization of drug toxicity *in vitro* before further consideration in human studies.

2.1. The current state-of-the-art of using hPSC for CNS safety screening

The extraordinary ability of hPSC to differentiate to CNS components makes them an interesting platform to better understand the deleterious effects of compounds on neural tissue [11, 22, 23]. Paired with cellular, genetic, biochemical, and functional assays, hPSC-derived neural tissue can be used to generate a comprehensive toxicological profile of drugs on the CNS and help to address decisions of go/no-go during a drug development process. Moreover, the brain undergoes significant postnatal development and its structure and function differ significantly between infantile and adult stages. Many drugs can affect the CNS differently, depending on the maturity of the subject (i.e., embryonic, infantile, or adult). Taken together, researchers may leverage hPSC-derived neural cells in different stages of differentiation to explore the safety profile of drugs on mature and immature nervous systems.

Neural precursor cells (NPCs) are multipotent cells, with the potential to generate multiple mature CNS cells, such as neurons, astrocytes, and oligodendrocytes [2, 24]. They are able to self-renew and proliferate, being pivotal players in the developing human brain. Toxicity to these cells at young stages of development can predispose the CNS to the onset of neurodevelopmental disorders and neurological impairments [25]. NPCs can be expanded *in vitro*, which makes them amenable to incorporation into large-scale studies. Protocols to maintain and differentiate NPCs in their CNS derivatives are well established, with great consistency and reproducibility [2, 26]. Moreover, hiPSC from different individuals can be used to obtain a progenitor cell bank representative of genetic differences found on our population. Assessing toxicological profile in such a heterogeneous genomic population could improve predictability of safety profiles of drugs on different individuals.

Publications with human NPCs started to demonstrate their use in assessing toxicological profile of drugs [25, 27]. Using hESC-derived NPCs, a research group described a platform for detection of toxicity to neuronal induction in embryonic development [28]. Researchers

exposed differentiating cells to methylmercury (MeHg) and found that it could disrupt early stages of neural differentiation. In another approach, researchers described the use of hESC-derived NPCs in identifying compounds that were selectively toxic to progenitor, but innocuous to terminally differentiated cells (neurons and astrocytes) [29]. Although the work was primarily envisioned as a platform to identify compounds able to deplete proliferation cells from heterogeneous neural populations *in vitro*, with applications in purifying populations for regenerative medicine, similar approach could also be used to elucidate the safety profile of chemical compounds on the CNS.

Differentiated populations of neurons have also been used to assess the toxicological profile of compounds [11]. Phenotypic assays such as neurite outgrowth and neuronal morphology have been used to investigate the effect of chemical entities on these populations. In a recent study, a library of 80 compounds was screened for their ability to inhibit neurite outgrowth in iPSC-derived neurons using a high-content screening platform [30]. From the compounds tested, 16 selectively inhibited neurite outgrowth, confirming the usefulness of hiPSC-derived neurons in neurotoxicity screenings. Although this study represents a step forward in developing a relevant humanized safety screening platform, it still relies on dissociated neurons plated at low density *in vitro*, which does not represent well the developed brain. Additionally, more sophisticated platforms, able to capture functional phenotypes, such as electrical activity of the neural circuitry, and the interplay between different CNS cell types will greatly help to improve the predictability of safety screenings.

2.2. Neural culture *in vitro*: from traditional tissue culture to organized organoids

Given the easy accessibility to CNS cells that hPSC offer, we have witnessed in the recent years the rediscovery of three-dimensional (3D) cell culture technologies as a powerful tool to study the brain [31, 32]. Organoids are 3D agglomerates of tissue-specific cells self-organized in structures that more closely resemble the target organ. Once organoids exhibit key structural and functional properties of a target tissue, they hold great promise in advancing the studies of complex organs such as the brain, where the interaction of many different cells organized in a defined structure is pivotal for its functions (**Figure 2**). Moreover, giving the complex interplay between neuronal and non-neuronal cells in the CNS, the deleterious effect of drugs may rely on non-neuronal cells (and not directly on neurons) but still lead to a pronounced effect on the nervous system. In this scenario, brain organoids could better capture any deviation from the homeostatic balance of the interaction between different neural cells.

Two different types of cells can be used to obtain organoids: hPSC (using either ESC or iPSC) or multipotent adult stem cells [32]. Both approaches rely on the potential for expansion and self-organization of these precursors *in vitro*. Many recent studies have confirmed the used of brain organoids in the modeling of diseases by recapitulating *in vitro* the intricate and complex processes occurring during human brain development. Moreover, brain organoid constructs incorporating many different cell types (such as neurons, astrocytes, endothelial, and microglia) can be obtained and used in developmental neurotoxicity screenings [33]. By using the described model, a recent study assessed the neurotoxicity profile of a library of 60 compounds and correctly classified 9 of 10 chemicals. Although organoids containing different tissues would be a model more representative of the organ *in vivo*, the different tissue-specific

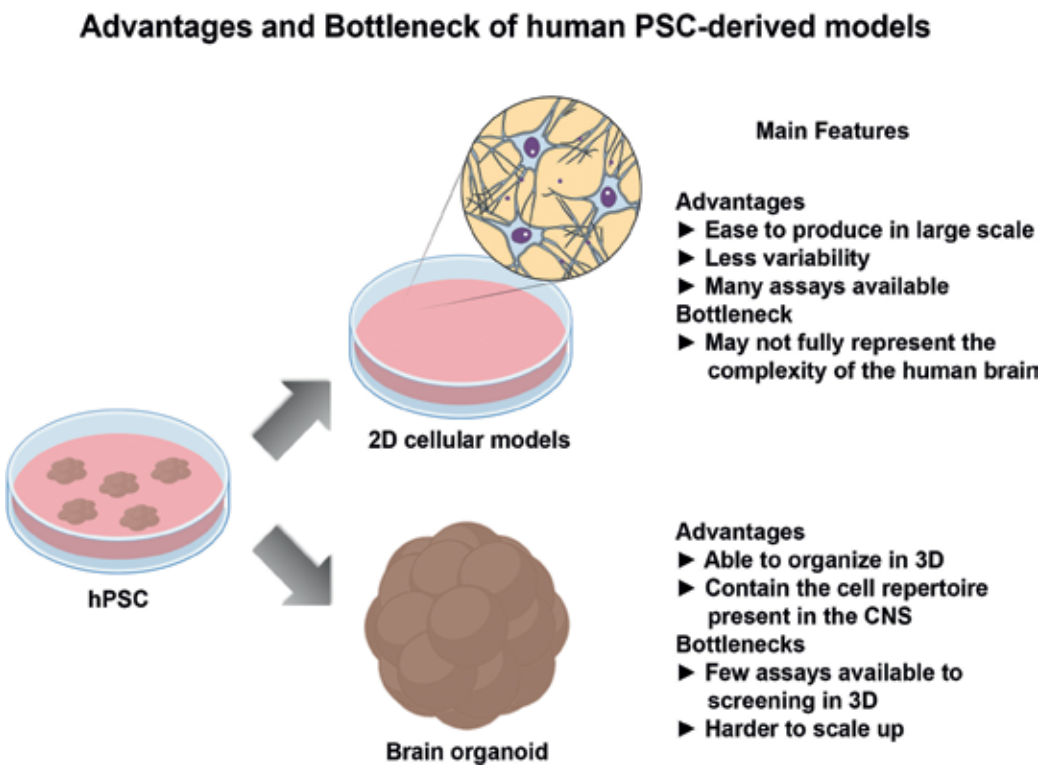

Figure 2. *In vitro* models derived from hPSC. The main advantages and bottlenecks are listed. Brain organoids are 3D structures derived from hPSC differentiation toward neuroectoderm.

cells lack the structural organization found *in vivo* and may not fully recapitulate the organ function or multi-tissue interactions.

3. Using MEA technology to evaluate neuroactivity of new chemicals

Cell viability, gene expression and neurite outgrowth assays with neurons have been the standard methods *in vitro* to measure deleterious effects of compounds on the CNS. In spite of their importance, they have limited predictability, especially with drugs able to evoke a deleterious functional change but without noticeable biochemical or cellular changes. Electrophysiology techniques exploit ionic conductance of ion channels and transient modulation of the membrane potential of a neuron, being able to assess the functional status of the neural network *in vitro* [34]. There are many different techniques to record neuronal activity *in vitro*, with the most commonly used being the patch-clamp method. Although very sensitive, its low throughput and limitation of assessing only one or few neurons at a time precludes its use in investigating large neuronal circuitry dynamics [34]. Recently, new technologies of extracellular recordings have been developed. They have many advantages over traditional patch-clamp techniques, such as being noninvasive, capable of monitoring the culture for long periods of time, and

able to record multiple cells at once, allowing large-scale assessment of neuronal circuitry dynamics [35]. This allows their use to better understand neuronal communication, information encoding, propagation, processing, and computation of neuronal circuits *in vitro* [36].

One of the most promising technologies to record extracellular signaling is the microelectrode arrays (MEA). Uniquely, MEA platforms consist of hundreds to thousands of electrodes integrated in a cell culture dish and enable recordings of neural activity by sensing extracellular field potentials [37]. This technology has been used to investigate the neural network dynamics of hESC- and hiPSC-derived neuronal cultures, organotypic slice cultures and acute brain slices [34]. By combining multiple arrays, the MEA technology allows to investigate several conditions at the time in a high-throughput fashion. Moreover, because MEA is a noninvasive technology and the neurons are cultured directly onto the electrodes, this technology enables the repeated monitoring of intrinsic and inducible changes in neuronal network dynamics for several days which is extremely useful to investigate the relative effects of chronic drug exposure in a dish [35, 38].

Functional electrophysiology of neurons represents a powerful tool to investigate the safety pharmacology of drugs prior to first-in-human studies. It needs to be noted though that the human brain contains hundreds of different types of neurons, each with unique properties and pharmacological signal transduction pathways which may not be fully recapitulated *in vitro*. When mimicking the human brain *in vitro*, it is imperative to select the most appropriate cellular model to ensure unequivocally adequate and highly reproducible predictability [34]. Although mouse and rat primary neuronal cultures are the gold standard in MEA electrophysiology, interspecies differences in ion-channel expression profile and neuronal response can be significantly different; therefore, translation of data to the human brain is very challenging in many situations [39]. The potential of using human PSC as a source of neuronal circuitry mimicking the human brain just started to be explored [40]. While preliminary results are confirming the use of hiPSC-derived neural culture as a powerful tool to explore neurotoxicity of compounds on the human brain, more studies are warranted to address the variability and heterogeneity of such cell culture models.

4. Overcoming current bottlenecks of hiPSC-derived cultures

Giving the high degree of complexity of the CNS cellular components, full translation from *in vitro* studies of compound-induced neurotoxicity can be challenging [40]. In the recent years, the stem cell field has produced diverse protocols for obtaining hiPSC-derived neurons *in vitro*, making any attempt to standardize the field complicate once each laboratory uses its own protocol. Moreover, the field is also subjected to batch-to-batch variation and long period of time differentiation protocols, which introduce additional challenges in reproducibility and hampers its full adoption by screening companies. Recently, however, a number of hiPSC-derived neurons became commercially available. Homogenous populations of neurons with specific neurotransmitter profiles are an attractive alternative to study the human physiology. The reduced variability from batch-to-batch and controlled differentiation process make possible the reproducible use of these cells to investigate neurotoxicity on the CNS. One thing to be noted though

is the difference between hiPSC-derived products offered by different companies. Additionally, while most companies focus on highly pure populations of neurons, depleted of glia cells, this model may not be ideal to mimic the CNS complexity. The presence of astrocytes, for example, are important to modulate the response of neurons to neurotransmitters and can affect the vulnerability of neuronal cultures to toxic insults [41, 42]. Moreover, co-culture with astrocytes enhances synaptic maturation, with consequences on firing frequency and bursting behavior [43, 44]. Although still an emerging field with many questions to be answered, commercially available hiPSC-derived neurons and astrocytes will be pivotal in validating this model as a suitable solution to reduce (or even replace) animal experimentation in toxicology studies.

5. Conventional drug development process and Zika virus

In February of 2016 the World Health Organization (WHO) declared the Zika virus infection a Public Health Emergency of International Concern (PHEIC), which prompted scientist worldwide to an urgent and coordinated response to this new global threat. Zika virus was first identified in 1947, but only recently received public attention after being associated with microcephaly in newborns and Guillain-Barré syndrome in adults [45, 46]. Two recent studies screened libraries of FDA-approved drugs and identified potential therapeutics with novel activity against the Zika virus [47, 48]. Repurposing FDA-approved drugs can potentially accelerate the discovery of cures to diseases, reducing time, and costs. However, both studies lack extensive neurotoxicity characterization of the potential therapeutics. Although the library consisted of FDA-approved compounds, the active concentrations against the virus were relatively high and not necessarily safe to human use. Moreover, it is pivotal to assess the safety of these compounds in early stages of the CNS development before considering them to treat pregnant women or newborns. In a recent scientific communication, our group demonstrated that many of the compounds identified on the mentioned studies were in fact toxic at their effective concentrations against Zika virus [49]. We investigated the toxicological profile of 29 compounds described as potential therapeutic against Zika virus infection. By testing hiPSC-derived cells at different stages of the CNS development, we observed greater toxicity at early stages of the nervous system, with decreasing toxicity as the cells matured *in vitro*. Interestingly, Emricasan (a compound highlighted in a previous publication) demonstrated a safe toxicological profile in all stages of the CNS and did not interfere with the normal function of mature neural cultures, as assessed by calcium mobilization assays using a fluorescent imaging plate reader (FLIPR) platform and electrophysiology using MEA [48]. Nonetheless, this study emphasized the need for extensive early characterization of repurposed compounds before considering them to potentially alleviate new diseases.

6. Conclusion/remarks

There is an urgency to accelerate and streamline the process of the development of new drugs. From devastating neurodegenerative disorders, such as Alzheimer's, to global threats,

epidemics from known and unknown viruses, we need to be able to rapidly identify safe therapeutic compounds. The average time to translate a drug from the bench to the clinic is 10 years, with an approximate cost of $2.6 billion dollars. One contributor for this is the fact that the current drug development process is very inefficient, with fewer than 10% of the drugs in development being approved for use [50]. Adverse drug reactions to CNS are responsible for a large amount of all drug attrition cases [51]. To change the current scenario, it is crucial to have available a toolbox able to quickly assess the toxicological profile in early steps of drug development. The incredible potential of hPSC to expand *in vitro* and differentiate toward any adult cell type makes them ideal tools to large-scale toxicology studies. Together with techniques able to assess functional phenotype in real time, such as MEA technology, terminally differentiated neurons derived from hPSC could help to improve clinical outcome predictability in early steps of the clinical trial, reducing overall costs and turnover of the drug development process.

In an attempt to streamline the discovery, development, and delivery of new cures, the House of Representatives of the United States of America recently passed the 21st Century Cures Act [52]. The bill will allocate funds to the National Institute of Health (NIH) and help to fast track the approval of new drugs by the Food and Drug Administration (FDA). The Cures Act will also provide funding for three innovative scientific initiatives: the Brain Research through Advancing Innovative Neurotechnologies (BRAIN) Initiative, the Precision Medicine Initiative (PMI) and the Beau Biden Cancer Moonshot Initiative [53]. Moreover, the Cures Act also simplifies the process of data sharing, allowing the quick use of data by the scientific community. The BRAIN initiative aims to elucidate how the neural network works in health brains and what is altered in neurological disorders. The building of knowledge on these brain states is pivotal to any drug development workflow. However, although initiatives as the BRAIN are required to advance our medical knowledge about the CNS, it is crucial to develop new platforms able to recapitulate these findings *in vitro*. To this end, platforms to assess adverse drug effects on the CNS using hiPSC are pointed as the most promising and currently being developed [17].

Human iPSC already started to revolutionize disease modeling *in vitro*, revealing disease mechanisms otherwise not seen using classical animal models. Moreover, once hiPSC can be derived from any individual, it enables their use in personalized medicine, including toxicological screening in individual-specific tissues to reveal the potential side effects of drugs before their use. One caveat though is the simplified representation of the nervous system tissue architecture that can be obtained in vitro using hiPSC-derived cell culture techniques. In an attempt to overcome this limitation, the field is seen as a re-emergence of 3D organoids. Recent studies with brain organoids have confirmed their potential in recapitulating steps of the human brain development and organization. The development of new 3D high-content screening technologies, such as Light Sheet Microscopy, and improved differentiation protocols will be critical to a broad adoption of this technology in drug development screenings. Moreover, they can be an attractive alternative in replacing animal use in certain applications as well in guiding conventional clinical trial studies for dose tolerance in humans. In principle, the use of brain organoids in screenings could help to provide a more fine-tuned and multipronged approach to understand the risks and benefits of new therapies [10].

In addition to hPSC, the repurposing of old drugs to new diseases have gained attention in the recent years and promise to revolutionize the drug discovery field [54]. Repurposing drugs could significantly decrease the time and costs to find new therapies. However, it is still crucial to re-evaluate their toxicological profile. When redirecting compounds to new diseases, their new efficacy dosage need to be extensively tested to assure safety on the clinic, once many redirected compounds present a higher effective concentration for 50% of the maximum response (IC_{50}) and may not be clinically relevant. The recent example of repurposing drugs to Zika virus found many hits with a higher IC_{50} than the safest dosage identified in a toxicological screening using hiPSC-derived neural progenitor cells, preventing their use in newborns and pregnant women [48, 49]. Drug combination therapy, using two or three compounds found in the repurposing screening, could potentially increase the success rate of such screening by synergistic effects of the combination [54]. Successfully synergistic combinations of drugs would enable the reduction of each drug dosage to nontoxic levels and allow to use a therapeutic concentration that is below or equal to their achievable human blood concentrations.

Finally, the Zika virus prompted the scientific community to react and collaborate in a fashion not seen before. Different fields joined forces sharing a common goal: discover new therapies and vaccines to an emerging global threat. It also highlighted the need to change the current drug development workflow. In face of such threats, new tools are needed allowing researchers to quickly identify new therapeutic compounds. Elements discussed on this chapter, such as hPSCs and their derivatives, combined with MEA electrophysiology will streamline this process and be the standard toxicological assays in the future.

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References

[1] Fields RD, Woo DH, Basser PJ. Glial regulation of the neuronal connectome through local and long-distant communication. Neuron. 2015;**86**(2):374-386. DOI: 10.1016/j.neuron.2015.01.014

[2] Carromeu C, Vessoni A, Diniz Mendes AP, Beltrão-Braga PCB. Differentiation of human pluripotent stem cells into cortical neurons. In: Ulrich H, Negraes PD, editors. Working with Stem Cells. 1st ed. Springer International, AG, Switzerland; 2016. pp. 163-180. DOI: 10.1007/978-3-319-30582-0

[3] BIO. Clinical Development Success Rates 2006-2015 [Internet]. 2016. Available from: https://www.bio.org/sites/default/files/Clinical%20Development%20Success%20Rates%202006-2015%20-%20BIO,%20Biomedtracker,%20Amplion%202016.pdf [Accessed: January 29, 2017]

[4] Dolmetsch R, Geschwind DH. The human brain in a dish: The promise of iPSC-derived neurons. Cell. 2011;**145**(6):831-834. DOI: 10.1016/j.cell.2011.05.034

[5] Dragunow M. The adult human brain in preclinical drug development. Nature Reviews Drug Discovery. 2008;**7**(8):659-666. DOI: 10.1038/nrd2617

[6] Clowry G, Molnár Z, Rakic P. Renewed focus on the developing human neocortex. Journal of Anatomy. 2010;**217**(4):276-288. DOI: 10.1111/j.1469-7580.2010.01281.x

[7] Cummings JL, Morstorf T, Zhong K. Alzheimer's disease drug-development pipeline: Few candidates, frequent failures. Alzheimer's Research & Therapy. 2014;**6**(4):37. DOI: 10.1186/alzrt269

[8] Thomas CE, Will Y. The impact of assay technology as applied to safety assessment in reducing compound attrition in drug discovery. Expert Opinion on Drug Discovery. 2012;**7:**109-122. DOI: 0.1517/17460441.2012.651122

[9] Carney EW, Settivari R. Predictive toxicology: Biological assay platform. In: Faqi AS, editor. A Comprehensive Guide to Toxicology in Preclinical Drug Development. 1st ed. Academic Press, Cambridge, MA; 2012. pp. 777-799

[10] Ko HC, Gelb BD. Concise review: Drug discovery in the age of the induced pluripotent stem cell. Stem Cells Translational Medicine. 2014;**3**(4):500-509. DOI: 10.5966/sctm.2013-0162

[11] Kolaja K. Stem cells and stem cell-derived tissues and their use in safety assessment. Journal of Biological Chemistry. 2014;**289**(8):4555-4561. DOI: 10.1074/jbc.R113.481028

[12] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science. 1998; **282**(5391):1145-1147. DOI: 10.1126/science.282.5391.1145

[13] NIH Human Embryonic Stem Cell Registry [Internet]. 2017. Available from: https://grants.nih.gov/stem_cells/registry/current.htm [Accessed: January 31, 2017]

[14] de Wert G, Mummery C. Human embryonic stem cells: Research, ethics and policy. Human Reproduction. 2003;**18**(4):672-682. DOI: 10.1093/humrep/deg143

[15] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;**126**(4):663-676. DOI: 10.1016/j.cell.2006.07.024

[16] Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;**131**(5):861-872. DOI: 10.1016/j.cell.2007.11.019

[17] Shi Y, Inoue H, Wu JC, Yamanaka S. Induced pluripotent stem cell technology: A decade of progress. Nature Reviews Drug Discovery. 2016 [Epub ahead of print]. DOI: 10.1038/nrd.2016.245

[18] Avior Y, Sagi I, Benvenisty N. Pluripotent stem cells in disease modelling and drug discovery. Nature Reviews Molecular Cell Biology. 2016;**17**(3):170-182. DOI: 10.1038/nrm.2015.27

[19] Russo FB, Cugola FR, Fernandes IR, Pignatari GC, Beltrão-Braga PC. Induced pluripotent stem cells for modeling neurological disorders. World Journal of Transplantation. 2015;**5**(4):209-221. DOI: 10.5500/wjt.v5.i4.209

[20] Marchetto MC, Carromeu C, Acab A, Yu D, Yeo GW, Mu Y, Chen G, Gage FH, Muotri AR. A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. Cell. 2010;**143**(4):527-539. DOI: 10.1016/j.cell.2010.10.016

[21] Nageshappa S, Carromeu C, Trujillo CA, Mesci P, Espuny-Camacho I, Pasciuto E, Vanderhaeghen P, Verfaillie CM, Raitano S, Kumar A, Carvalho CM, Bagni C, Ramocki MB, Araujo BH, Torres LB, Lupski JR, Van Esch H, Muotri AR. Altered neuronal network and rescue in a human MECP2 duplication model. Molecular Psychiatry. 2016;**21**(2):178-188. DOI: 10.1038/mp.2015.128

[22] Ni M, Li X, Rocha JB, Farina M, Aschner M. Glia and methylmercury neurotoxicity. Journal of Toxicology and Environmental Health, Part A. 2012;**75**(16-17):1091-1101. DOI: 10.1080/15287394.2012.697840

[23] Farina M, Aschner M, Rocha JB. Oxidative stress in MeHg-induced neurotoxicity. Toxicology and Applied Pharmacology. 2011;**256**(3):405-417. DOI: 10.1016/j.taap.2011.05.001

[24] Seaberg RM, van der Kooy D. Stem and progenitor cells: The premature desertion of rigorous definitions. Trends in Neurosciences. 2003;**26**(3):125-131. DOI: 10.1016/S0166-2236(03)00031-6

[25] Tofighi R, Moors M, Bose R, Ibrahim WN, Ceccatelli S. Neural stem cells for developmental neurotoxicity studies. Methods in Molecular Biology. 2011;**758**:67-80. DOI: 10.1007/978-1-61779-170-3_5

[26] Muratore CR, Srikanth P, Callahan DG, Young-Pearse TL. Comparison and optimization of hiPSC forebrain cortical differentiation protocols. PLoS One. 2014;**9**(8):e105807. DOI: 10.1371/journal.pone.0105807

[27] Yap MS, Nathan KR, Yeo Y, Lim LW, Poh CL, Richards M, Lim WL, Othman I, Heng BC. Neural differentiation of human pluripotent stem cells for nontherapeutic applications: Toxicology, pharmacology, and In vitro disease modeling. Stem Cells International. 2015;**2015**:105172. DOI: 10.1155/2015/105172

[28] Stummann TC, Hareng L, Bremer S. Hazard assessment of methylmercury toxicity to neuronal induction in embryogenesis using human embryonic stem cells. Toxicology. 2009;**257**(3):117-126. DOI: 10.1016/j.tox.2008.12.018

[29] Han Y, Miller A, Mangada J, Liu Y, Swistowski A, Zhan M, Rao MS, Zeng X. Identification by automated screening of a small molecule that selectively eliminates neural stem cells derived from hESCs but not dopamine neurons. PLoS One. 2009;**4**(9):e7155. DOI: 10.1371/journal.pone.0007155

[30] Ryan KR, Sirenko O, Parham F, Hsieh JH, Cromwell EF, Tice RR, Behl M. Neurite outgrowth in human induced pluripotent stem cell-derived neurons as a high-throughput screen for developmental neurotoxicity or neurotoxicity. Neurotoxicology. 2016;**53**:271-281. DOI: 10.1016/j.neuro.2016.02.003

[31] Bredenoord AL, Clevers H, Knoblich JA. Human tissues in a dish: The research and ethical implications of organoid technology. Science. 2017;**355**(6322):pii: eaaf9414. DOI: 10.1126/science.aaf9414

[32] Lancaster MA, Knoblich JA. Organogenesis in a dish: Modeling development and disease using organoid technologies. Science. 2014;**345**(6194):1247125. DOI: 10.1126/science.1247125

[33] Schwartz MP, Hou Z, Propson NE, Zhang J, Engstrom CJ, Santos Costa V, Jiang P, Nguyen BK, Bolin JM, Daly W, Wang Y, Stewart R, Page CD, Murphy WL, Thomson JA. Human pluripotent stem cell-derived neural constructs for predicting neural toxicity. Proceedings of the National Academy of Sciences of the United States of America. 2015;**112**(40):12516-12521. DOI: 10.1073/pnas.1516645112

[34] Accardi MV, Pugsley MK, Forster R, Troncy E, Huang H, Authier S. The emerging role of in vitro electrophysiological methods in CNS safety pharmacology. Journal of Pharmacological and Toxicological Methods. 2016;**81**:47-59. DOI: 10.1016/j.vascn.2016.03.008

[35] Spira ME, Hai A. Multi-electrode array technologies for neuroscience and cardiology. Nature Nanotechnology. 2013;**8**(2):83-94. DOI: 10.1038/nnano.2012.265

[36] Obien ME, Deligkaris K, Bullmann T, Bakkum DJ, Frey U. Revealing neuronal function through microelectrode array recordings. Frontiers in Neuroscience. 2015;**8**:423. DOI: 10.3389/fnins.2014.00423

[37] Illes S, Fleischer W, Siebler M, Hartung HP, Dihné M. Development and pharmacological modulation of embryonic stem cell-derived neuronal network activity. Experimental Neurology. 2007;**207**(1):171-176. DOI: 10.1016/j.expneurol.2007.05.020

[38] Johnstone AF, Gross GW, Weiss DG, Schroeder OH, Gramowski A, Shafer TJ. Microelectrode arrays: A physiologically based neurotoxicity testing platform for the 21st century. Neurotoxicology. 2010;**31**(4):331-350. DOI: 10.1016/j.neuro.2010.04.001

[39] Atack JR, Wafford KA, Street LJ, Dawson GR, Tye S, Van Laere K, Bormans G, Sanabria-Bohórquez SM, De Lepeleire I, de Hoon JN, Van Hecken A, Burns HD, McKernan

RM, Murphy MG, Hargreaves RJ. MRK-409 (MK-0343), a GABAA receptor subtype-selective partial agonist, is a non-sedating anxiolytic in preclinical species but causes sedation in humans. Journal of Psychopharmacology. 2011;**25**(3):314-328. DOI: 10.1177/0269881109354927

[40] Tukker AM, de Groot MW, Wijnolts FM, Kasteel EE, Hondebrink L, Westerink RH. Is the time right for in vitro neurotoxicity testing using human iPSC-derived neurons? ALTEX. 2016;**33**(3):261-271. DOI: 10.14573/altex.1510091

[41] Beaman-Hall CM, Leahy JC, Benmansour S, Vallano ML. Glia modulate NMDA-mediated signaling in primary cultures of cerebellar granule cells. Journal of Neurochemistry. 1998;**71**(5):1993-2005. DOI: 10.1046/j.1471-4159.1998.71051993.x

[42] Dugan LL, Bruno VM, Amagasu SM, Giffard RG. Glia modulate the response of murine cortical neurons to excitotoxicity: Glia exacerbate AMPA neurotoxicity. The Journal of Neuroscience. 1995;**15**(6):4545-4555

[43] Clarke LE, Barres BA. Emerging roles of astrocytes in neural circuit development. Nature Reviews Neuroscience. 2013;**14**(5):311-321. DOI: 10.1038/nrn3484

[44] Odawara A, Saitoh Y, Alhebshi AH, Gotoh M, Suzuki I. Long-term electrophysiological activity and pharmacological response of a human induced pluripotent stem cell-derived neuron and astrocyte co-culture. Biochemical and Biophysical Research Communications. 2014;**443**(4):1176-1181. DOI: 10.1016/j.bbrc.2013.12.142

[45] Dick GW, Kitchen SF, Haddow AJ. Zika virus. I. Isolations and serological specificity. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1952;**46**(5):509-520. DOI: 10.1016/0035-9203(52)90042-4

[46] Pierson TC, Graham BS. Zika virus: Immunity and vaccine development. Cell. 2016; **167**(3):625-631. DOI: 10.1016/j.cell.2016.09.020

[47] Barrows NJ, Campos RK, Powell ST, Prasanth KR, Schott-Lerner G, Soto-Acosta R, Galarza-Muñoz G, McGrath EL, Urrabaz-Garza R, Gao J, Wu P, Menon R, Saade G, Fernandez-Salas I, Rossi SL, Vasilakis N, Routh A, Bradrick SS, Garcia-Blanco MA. A screen of FDA-approved drugs for inhibitors of Zika virus infection. Cell Host & Microbe. 2016;**20**(2):259-270. DOI: 10.1016/j.chom.2016.07.004

[48] Xu M, Lee EM, Wen Z, Cheng Y, Huang WK, Qian X, Tcw J, Kouznetsova J, Ogden SC, Hammack C, Jacob F, Nguyen HN, Itkin M, Hanna C, Shinn P, Allen C, Michael SG, Simeonov A, Huang W, Christian KM, Goate A, Brennand KJ, Huang R, Xia M, Ming GL, Zheng W, Song H, Tang H. Identification of small-molecule inhibitors of Zika virus infection and induced neural cell death via a drug repurposing screen. Nature Medicine. 2016;**22**(10):1101-1107. DOI: 10.1038/nm.4184

[49] Society for Laboratory Automation and Screening (SLAS) Conference 2017 [Internet]. 2017. Available from: https://www.eventscribe.com/2017/SLAS2017/ajaxcalls/postersinfo.asp?title=84821 [Accessed: February 11, 2017]

[50] The Scientist [Internet]. 2017. Available from: http://www.the-scientist.com/?articles.view/articleNo/48280/title/Opinion-Improving-FDA-Evaluations-Without-Jeopardizing-Safety-and-Efficacy/ [Accessed: February 3, 2017]

[51] Arrowsmith J, Miller P. Trial watch: Phase II and phase III attrition rates 2011-2012. Nature Reviews Drug Discovery. 2013;**12**(8):569. DOI: 10.1038/nrd4090

[52] The Scientist [Internet]. 2016. Available from: http://www.the-scientist.com/?articles.view/articleNo/47639/title/Biomedical-Research-Bill-Approved-in-House/ [Accessed: February 1, 2017]

[53] Hudson KL, Collins FS. The 21st century cures Act – A view from the NIH. The New England Journal of Medicine. 2017;**376**(2):111-113. DOI: 10.1056/NEJMp1615745

[54] Sun W, Sanderson PE, Zheng W. Drug combination therapy increases successful drug repositioning. Drug Discovery Today. 2016;**21**(7):1189-1195. DOI: 10.1016/j.drudis.2016.05.015

Chapter 2

Induced Pluripotent Stem Cell-Derived Human Glutamatergic Neurons as a Platform for Mechanistic Assessment of Inducible Excitotoxicity in Drug Discovery

Yafei Chen

Additional information is available at the end of the chapter

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Abstract

Since the guiding principles of Replace, Reduce, and Refine were published, wider context-of-use for alternatives to animal testing have emerged. Induced pluripotent stem cell-derived human glutamatergic-enriched cortical neurons can be leveraged as 2- and 3-dimensional platforms to enable candidate drug screening. Uniquely so, 2-dimensional models are useful considering that they exhibit spontaneous firing, while, 3-dimensional models show spontaneous synchronized calcium transient oscillations. Here, the limitations of selected induced acute seizure models as well as the early utilization of fully differentiated glutamatergic neuron models for interrogation of inducible excitotoxicity following exposure to neuromodulators will be described. The context of use for candidate biomarkers of inducible seizure is also discussed.

Keywords: GlutaNeurons, microelectrode array, fluorometric imaging plate reader

1. Introduction

Animal models are often leveraged to evaluate neuronal function within an intact system [1]; yet, the "gold standard" of ex vivo evaluation of brain pathophysiology is "the brain slice assay" [2]. Brain slice models lend to reduced numbers of animals for the conduct of neuroscience research. While primary neuronal cell cultures derived from animals have supported *in vitro* neuroscience studies for decades, their utility often leave to question possible translation to humans [3]. Human embryonic stem cell models were envisioned to provide more

translatable context [4]. However, ethical bias towards stem cell procurement from embryos and the lack of access to physiologically-relevant adult human brain tissues further encouraged scientists to focus on the advancement of human induced pluripotent stem cell (iPSC)-derived neuronal models [5]. Several iPSC-derived neurons have been developed to recapitulate network behavior and signaling cascades on-a-dish reminiscent of phenotypes observed in humans. Comprising the predominant excitatory network are glutamatergic neurons throughout the cortex, cerebellum, hippocampus, striatum, thalamus, hypothalamus, and visual/auditory system. Neuronal vesicles contain glutamate and migrate to the synapse where they release glutamate into the synaptic gap following Na^+/K^+ exchange (depolarization). More recently, iPSC-derived human glutamatergic neurons were generated to support cell-based research strategies [6]. Hence, researchers continue to learn and integrate these cutting-edge technologies for early safety assessment. This chapter will spotlight the validation of glutamatergic neurons as a platform for mechanistic assessment of inducible excitotoxicity in drug discovery.

2. Challenges when leveraging iPSC-derived human glutamatergic neurons

The most important tasks in using iPSC-derived human glutamatergic neurons is achieving large-scale generation of human iPSC-derived neural stem cells/early neural progenitor cells in spite of several challenges related to the differentiation procedures [7]. Terminal differentiation coupled with preliminary cell model qualification steps often reveal how different iPSC lines made from a single donor exhibit similar phenotypic marker expression as well as sensitivity to selected neurotoxicants. Yet, a limitation of iPSCs, in general, is lot-to-lot variability, including optimizations of culturing conditions (e.g. medium, supplements), following advanced understanding of how to optimal use such models to assess compound-related effects *in vitro*. The iCell® GlutaNeurons (Cellular Dynamics International, a Fujifilm Company, cat. no. GNC-301-030-001) were developed to provide a more physiologically relevant *in vitro* platform for de-risking potentially translatable compound-induced neuronal toxicity early in the drug discovery process. Notably, iCell® GlutaNeurons represent post-mitotic and highly pure populations (≥90%) of human cortical glutamatergic neurons (**Figure 1**). According to the manufacturer, the iPS cell lines were generated from human peripheral blood through ectopic expression of reprogramming factors by episomal transfection. The iPS cell clones were engineered using nuclease-mediated methodologies to exhibit neomycin resistance under the control of a neuronal-specific promoter.

When considering the impact of epigenetic factors state in neuronal development and differentiation and that neurons derived from iPSCs offer a consistently reliable source of cells from a single donor, baseline gene expression analysis profiles for novel iPSC-derived human glutamatergic neurons represents another paramount validation step. Baseline single cell gene transcription analyses was determined from iCell® GlutaNeurons at DIV7 and DIV21 using a Biomark HD platform and custom assay reagents purchased from Fluidigm (**Figure 2**).

In general, a major challenge when leveraging hiPSC-derived GlutaNeurons is the lack of endogenous mechanisms for detoxification as well as the ability to evaluate the impact of different routes of administration (e.g., intramuscular and/or intraperitoneal injections) and the inability to evaluate relevant co-morbidities which are observed in animal models and/or

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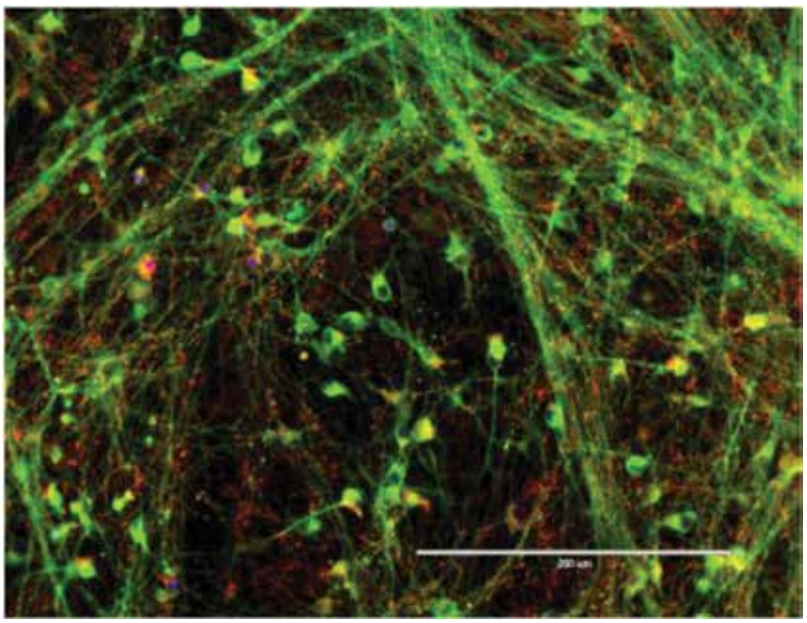

Figure 1. Human iCell® GlutaNeurons at DIV14. Fluorescence immunolabeling for phenotypic marker indicated neurons (βIII-tubulin stain, green); non-neoplastic neurons (synaptophysin stain, red) and non-neuronal cells (4′,6-diamidino-2-phenylindole, DAPI stain, blue). Branched networks were observed rapidly (after 24 h) and remained viable and adherent for an extended period in culture.

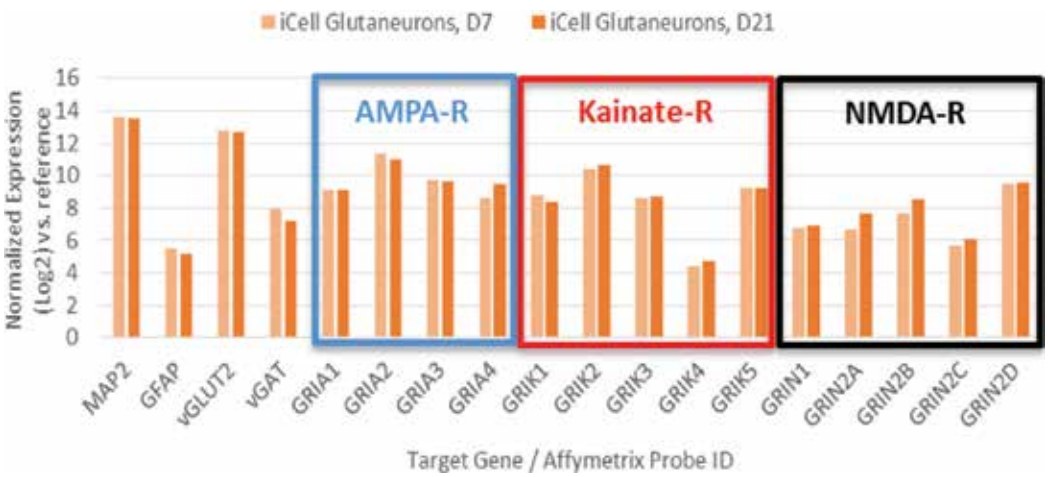

Figure 2. Targeted single-cell analysis to characterize gene transcription in untreated iCell® GlutaNeurons at DIV7 and DIV21. We found that most cells were positive for glutamatergic gene transcripts in the absence of differential expression at the two different time points. Abbreviations: GRIA—glutamate receptor, ionotropic, AMPA; GRIK—glutamate receptor, ionotropic, kainate; GRIN—glutamate receptor, ionotropic, NMDA.

humans. Therefore, initial GlutaNeuron assessments rely on paradigm compounds that have been previously well-characterized *in vivo* [8]. While they provide a way to model *in vitro* glutamate excitotoxicity, iPSC-derived GlutaNeurons do not enable the determine relationships between compound and brain segment-specific toxicological responses [9].

3. Glutamate excitotoxicity

There are three major classes of ionotropic glutamate receptors (iGluRs) and eight subtypes of metabotropic glutamate receptors (mGluRs) [10]. The iGluRs are predominantly located in postsynaptic sites and mediate rapid excitatory transmission, while mGluRs are expressed in both neurons and glial cells and mediate delayed transmission. The amino acid L-glutamate functions as an excitatory neurotransmitter that preferentially binds to iGluRs and mGluRs on postsynaptic neurons which follows depolarization by Na^+, K^+, and Ca^{2+}, resulting in an action potential [11]. Conversely, the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) functions to result in hyperpolarization of postsynaptic neurons [12]. Glutamate is synthesized in neurons, while excessive glutamate is take up from the synaptic cleft by glial cells (astrocytes) via excitatory amino acids transporters EAAT1 and EAAT2 [13, 14]. In astrocytes, glutamate is converted to glutamine by the enzyme glutamate synthase (GS). The glutamine is transported back into presynaptic terminals via the "neuronal" excitatory amino acids transporter EAAT3, where it is transformed by the glutaminase to yield glutamate that returns to vesicles via vesicular glutamate transporters (vGLUT1 and vGLUT2). This biochemical process is referred to as "the glutamate-glutamine cycle" [15]. Activation of mGluRs by glutamate leads to intracellular inositol trisphosphate (IP3) turnover, activation of ryanodine receptor channels in the endoplasmic reticulum (ER) membrane, and subsequent release of Ca^{2+} from the ER into the cytoplasmic space. Increased cytoplasmic Ca^{2+} can induce mitochondrial uptake of Ca^{2+} which, if excessive, may result in the production of reactive oxygen species (ROS) caused by prostaglandin/leukotriene formation and/or insufficient intracellular antioxidants/free radical scavengers; production of reactive nitrogen species (RNS) and/or production of apoptogenic factors; activation of calcium dependent phospholipases, endonucleases, and proteases; untoward mitochondrial alterations (loss of buffering capacity); progressive neuronal excitotoxicity; caspase activation; ATP depletion; and ultimately neuronal apoptosis, necrosis and/or death [16–18]. Glutamate and structurally related analogs of glutamate can interact with N-methyl D-aspartic acid receptors (NMDAR) under membrane depolarization conditions (removal of intrinsic Mg^{2+} channel block of relative Ca^{2+}-dependent responses), resulting in large amounts of Ca^{2+} influx which activates downstream signal transduction cascade to produce neurotoxic damage. These effects can be abrogated by antagonists. Additionally, binding of glutamate to a-amino-3-hydroxy-5-methy-4-isoxazole propionic acid receptors (AMPAR) and/or kainate (KA) receptors (KAR) results in Na^+ influx, membrane depolarization and opening the voltage-dependent Ca^{2+} channels, signaling excitotoxicity. Conversely, minimal excitotoxicity may follow mitochondrial depolarization, leading to autophagy, a neuronal protective mechanism.

4. Kainate-induced rat status epilepticus model

In vitro platforms need to be coupled with *in vivo* models to establish translational relevance. For example, kainate acid (kainate or KA)-induced rat status epilepticus model (**Figure 3A**) is hallmarked by behavioral status epilepticus for up to 7 h following kainate injection coupled

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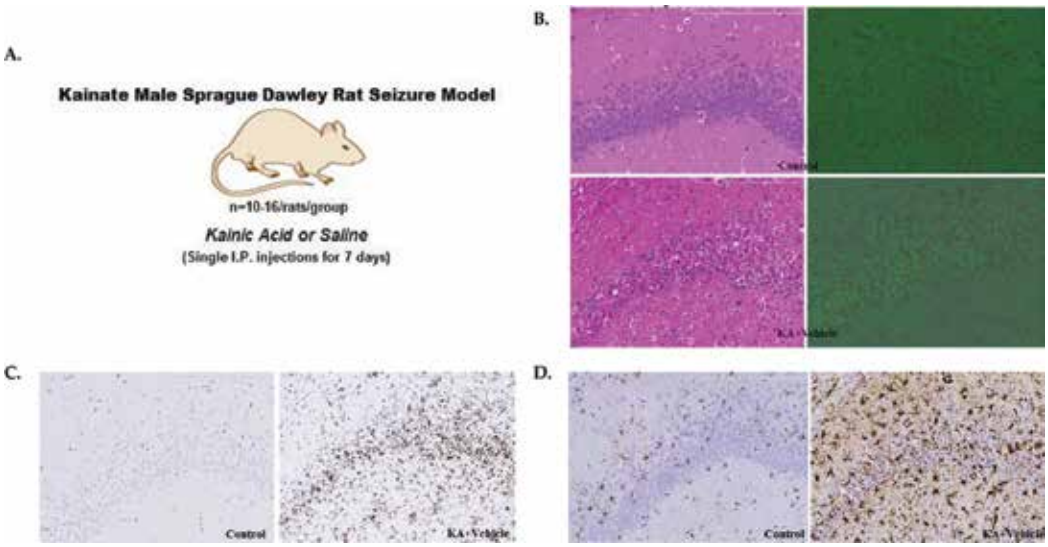

Figure 3. Hematoxylin and eosin staining and Fluoro-Jade B immunostaining in male Sprague Dawley rats that received once daily intraperitoneal (I.P.) injections of either vehicle (saline for injection USP, n = 10) or kainate (8 mg/kg/day, n = 16) for 7 consecutive days (A). In 9/16 KA-treated rats, mild to marked neuronal degeneration marked by increased Flouro-J immunolabeling when compared to controls (B), ED1/CD68 immunolabeling, indicating increased microglial activation (C) and GFAP immunolabeling, indicating increased astrogliosis (D) were identified in the hippocampus CA1-CA3 regions.

Kainate Related Brain Histopathology Findings (Day 8)

Hippocampal Lesion	Normal	Minimal	Mild	Moderate	Marked
None	1/16	10/16	10/16	10/16	10/16
CA1	1/16	0/16	0/16	0/16	0/16
CA1, CA3	0/16	0/16	1/16	0/16	3/16
CA1, CA2, CA3	0/16	0/16	0/16	0/16	4/16

Lesion severity scoring scheme: Normal (0 affected cells); 1-Minimal (1-100 affected cells); 2-Mild (101-200 affected cells); 3-Moderate (201-300 affected cells); and 4-Marked (>300 affected cells).

Table 1. Summary of brain histopathology finding in male Sprague Dawley rats that received once daily intraperitoneal injections of kainate (8 mg/kg/day, n = 16) for 7 consecutive days. Brain tissues were unremarkable from the control group rats that received vehicle (saline for injection USP, n = 10) for 7 consecutive days (data not shown).

with brain lesions characterized by mild to marked neuronal degeneration (**Table 1**, **Figure 3B**), microglial activation [increase in number] (**Figure 3C**) and astrogliosis (**Figure 3D**) in the hippocampus CA1–CA3 regions.

5. Kainate-induced neuronal toxicity in-a-dish

Kainate-induced experimental seizure in animals replicate features of human temporal lobe epilepsy (TLE), while neuronal toxicity in-a-dish models [19, 20] are useful because direct exposure of cells to specific concentrations of compound results in the establishment of EC50 or IC50 concentrations. These cell-based models are usually dispersed in a monolayer bed, with or without an astrocytic layer beneath it. Generally, 18-day-old fetal neurons are commonly used, and even "mature" for several weeks in culture, these neurons are not comparable to adult neurons *in vivo* [21]. The excitatory iCell® GlutaNeurons were derived using iPSC technology. Spontaneous electrical activity, including mean firing rat, was measured via microelectrode array (MEA) technology before and after reference compound application.

The human iCell® GlutaNeurons are differentiated from a master bank of stably iPSCs and provided as cryopreserved single-cell suspensions in 1.5 ml frozen cryovials (containing at least 5–6 million cells/vial) on dry ice. Upon receipt, the original vials were immediately transferred to liquid nitrogen tank storage until defrosted for cell culture according to the User's Guide provided by the vender. At the time of plating, the cells were thawed for exactly 2 min in a 37°C water bath. Complete BrainPhys medium comprised of the BrainPhys Neuronal Medium, iCell DopaNeurons Medium Supplement, iCell Nervous System Supplement, N-2 supplement, laminin, and penicillin-streptomycin. The Complete BrainPhys medium is serum free and has been specially formulated whereby it enables cell viability and function while limiting the proliferation of progenitor or non-neuronal cells. Cells were gently transferred to a 50 ml tube and 1 ml of the complete BrainPhys medium was added drop-wise and swirled gently to minimize osmotic shock while an additional 8 ml of BrainPhys medium was slowly added. The cell suspension was centrifuged at 400× g at room temperature for 5 min, carefully aspirate the supernatant, and resuspend the cell pellet in 2-3 ml of the complete BrainPhys medium after cell counting. The re-suspendered cells (100 μl/well) were seeded at a density of 4 × 104 cells/well (12.5×10^4 cell/cm^2) in a Corning 96-well white clear flat bottom polystyrene microplate (Corning, New York), or at a density of 8×10^4 cells/well in an Axion 48-well MEA plate (**Figure 4**).

Cells were maintained at 37°C (5% CO_2) in the complete BrainPhys medium as describe below. The cell culture plate well surfaces were freshly prepared with a base layer of Poly-L-ornithine solution (0.01%; Sigma Aldrich) and a top coating of Matrigel solution (0.028 mg/ml; Sigma-Aldrich), which are used to promote cell attachment, viability, and function. Spontaneous electrical activity was recorded as described previously [23]. In general, homogeneous neuronal populations produce spontaneous action potentials; therefore, mean firing rate (MFR) reflects the action potentials over time which can be used in part to quantify cell functionality. Multiple action potentials within a short time period represent neuronal burst. The cells were

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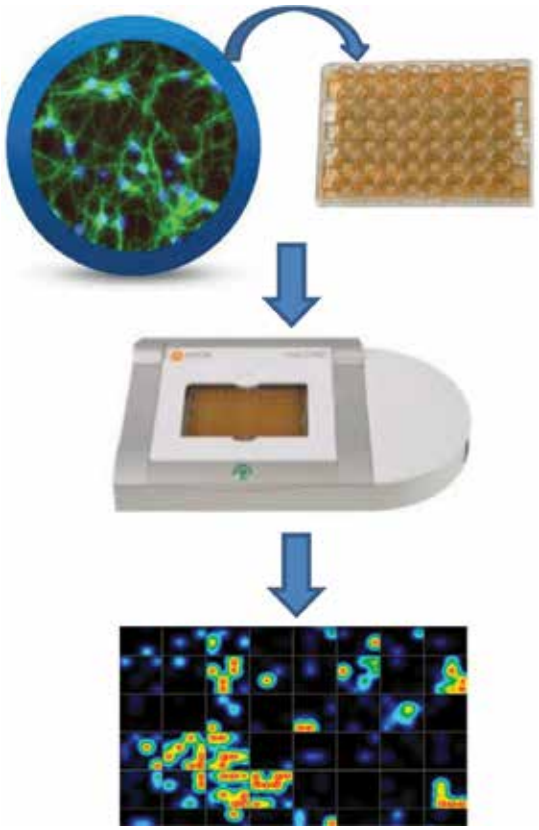

Figure 4. For example, the Axion classic 48-well MEA plates consist of wells with an array of 16 individually embedded, nanotextured gold microelectrodes with four integrated ground electrodes, yielding a total of 768 channels across the entire plate [22]. The culture plate temperature can be maintained at 37°C using an integrated heating system and temperature controller based on the low throughput maestro platform (Axion BioSystems, Inc.). The newer low throughput, maestro PRO and high throughput, Maestro APEX platforms from Axion BioSystems Inc. (not shown) may also be leveraged.

maintained at 37°C in an incubator with 5% CO_2 for several days *in vitro* (DIV) with exchange of 50% of the medium every 2 days before treatment and subsequent parameter measurements. Per our use, the iCell® GlutaNeurons were maintained in culture for up to 21 days in culture medium without appreciable loss of viability (data not shown).

We tested glutamate receptor agonists and antagonists iCell® GlutaNeurons at DIV9, using biochemical/functional assays to assess changes in cell viability, biomarker expression levels and synaptic activity to systematically evaluate concentration and time dependent glutamate excitotoxicity. Cells were treated with either vehicle (media or 0.1% DMSO), agonist (glutamate), kainate (positive control agonist) or antagonist (NMDA-R Antagonist, MK801; KA-R/AMPA-R Antagonist, NBQX; and FAAH Inhibitor, URB597) for up to 24 h, or pre-treated with glutamate receptor antagonists or FAAH inhibitor for 1 h followed by an agonist for up to 24 h. Changes in cell density were determined subjectively using routine bright field microscopic analysis (**Figure 5**). Concentration-dependent glutamate and kainate induced reductions

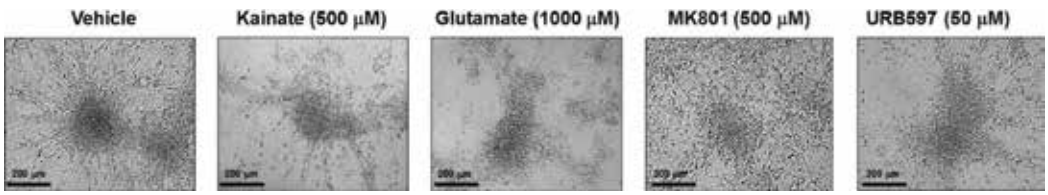

Figure 5. Changes in cell density of iCell® GlutaNeurons. Reductions in cell density were observed in GlutaNeuron-astrocyte co-cultures following exposure to kainate (B) when compared to vehicle (A). Phenotypic changes were apparent with glutamate only (C). Alone, no MK801- (D) or URB597- (E) related effects were observed. iCell® GlutaNeurons were imaged in brightfield using an EVOS XL digital inverted microscope.

in mean cell viability were assessed and compared at 30 min (acute effect; data not shown) and 24 h (lasting effect; **Figure 6**). For replicate wells for the same treatments, the mean values relative to concurrent controls were used to calculate the area under the concentration curve (AUC) in GraphPad Prism 7.0 software. After test compound treatment using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega Corporation, Madison,). Briefly, the CellTiter-Glo® reagent added directly to cells culture medium results in cell lysis and generation of a luminescent signal (by luciferase reaction) proportional to the amount of ATP, which is directly proportional to the number of viable cells present in culture. In this study, ATP levels released from viable cells mitochondrial were measured in the 96-well plate format with

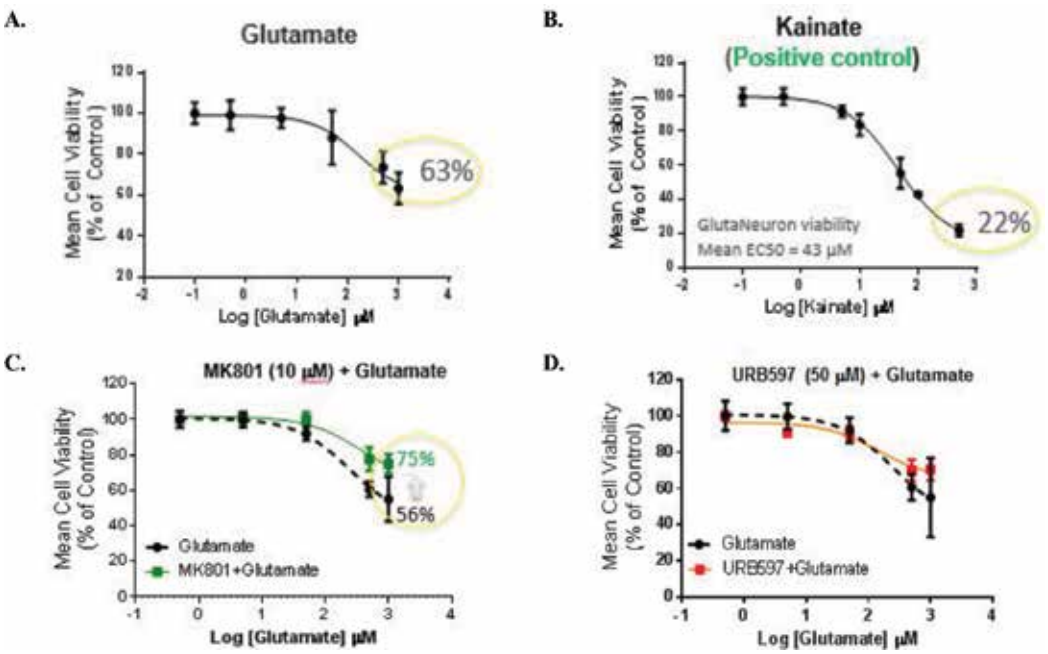

Figure 6. Mean cell viability of iCell® GlutaNeurons following exposure to glutamate (A, 63% loss) and kainate (B, 22% loss) for 24 h when compared to controls. MK801 (NMDA antagonist, 10 μM = IC50) partially blocked the glutamate-induced loss (~20%) of mean cell viability (C). FAAH inhibitor URB597, 50 mM) did not reduce the glutamate-induced effects (D).

approximately 4×10^4 or 8×10^4 cells/well 24 h after drug treatment using a microplate luminescence reader. All experiments were performed in triplicate wells for each condition (vehicle or per test compound concentration) and repeated at least three times. Blockade of KA-induced effects on mean cell viability by pre-treatment with different concentrations of NBQX was evident (data not shown).

The Axion BioSystems Maestro MEA technology is a label-free platform that measures electrical activity of iCell® GlutaNeurons when cultured directly on MEA plates. Established algorithms within Axion Integrated Studios (AxIS) v1.9 software can detect and quantify several parameters to assess changes in neuronal functionality, excitability and connectivity. On DIV10 and DIV16, spontaneous network activity was recorded using Axion Biosystems Maestro 768 channel amplifier and AxIS) v1.9 software. The amplifier recorded from all channels simultaneously using a gain of 1200× and a sampling rate of 12.5 kHz/channel. After passing the signal through a Butterworth band-pass filter (300–5000 Hz) on-line spike detection (threshold = 8× rms noise on each channel) was done with the AxIS adaptive spike detector. All recordings were conducted at 37°C. Spontaneous network activity was recorded in the absence of compounds for 4 min (baseline), then compounds were added and a continuous 30 min (acute effects), or various time points (4 min/time points) up to 24 h (sub-chronic effects) of activity were recorded. Only wells with 10 or more active electrodes (>5 spikes/min) during the

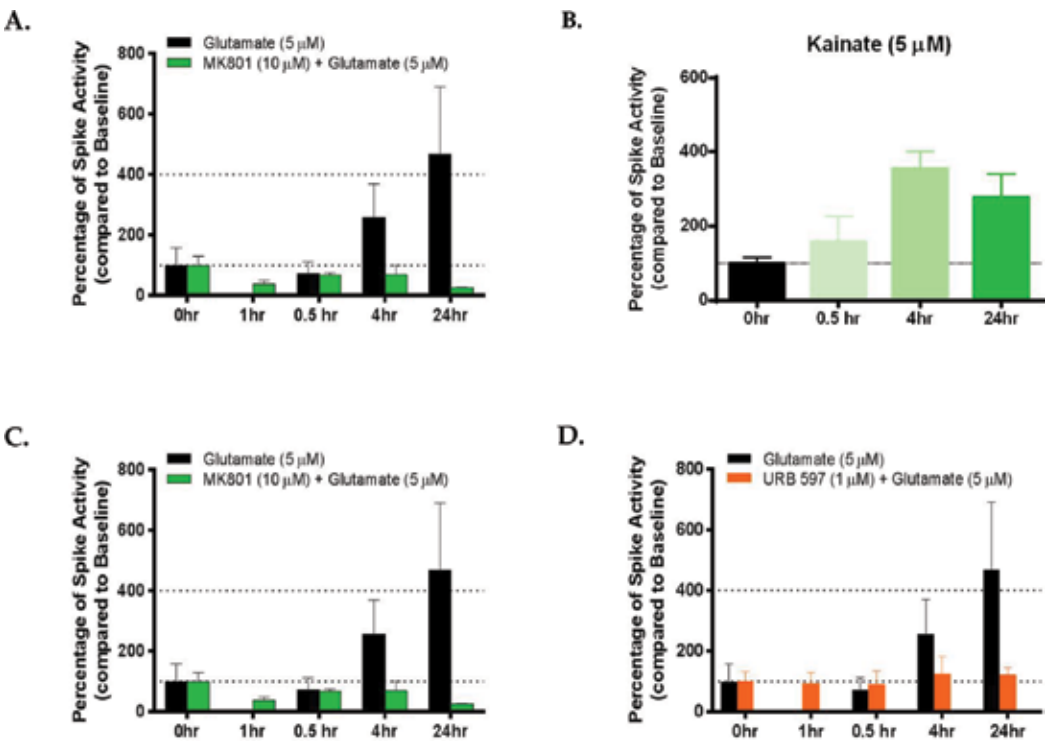

Figure 7. Kainate (A) and glutamate (B) induced changes in MFR in GlutaNeurons. MEA data showed that MK801 (10 μM) attenuated the glutamate (5 μM)-induced excitation at 4 and 24 h (C). Cells pre-exposed to URB597 reduced glutamate (5 μM)-induced excitation at 4 and 24 h in GlutaNeurons (D).

baseline recording period were used in the analysis. Stock solutions of compounds were made to 100× the desired final concentration in 100 µl media, then 2 µl of stock solution was added to each well (200 µl media) to reach the final desired concentration. Dedicated wells were used on each 48 well plate for vehicle controls. At least three replicates of the viability assay and were performed. At least triplicated wells were measured in each replicate. Following addition of neat compounds, electric activity was recorded continuously for 30 min to determine the acute effect on neuronal activity, or at different time points up to 24 h to determine the sub-chronic effect on neuronal activity. Briefly, neurons synaptic activities (spontaneous or induced action potentials) recorded in 4 min intervals at −2, 0 (baseline), 0.5, 2, 4 and 24 h post-treatment, and MFR was used a biomarker to quantify functional changes in GlutaNeurons. Between 0.5-24 h post treatment, pharmacologically relevant concentrations of kainate (**Figure 7A**) and Glutamate (**Figure 7B**) induced changes in MFR in GlutaNeurons. MEA data showed that MK801 (10 µM) attenuated the Glutamate (5 µM)-induced excitation at 4 and 24 h (**Figure 7C**). Cells pre-exposed to URB597 reduced Glutamate (5 µM)-induced excitation at 4 and 24 h in GlutaNeurons (**Figure 7D**).

6. iPSC-derived neuronal models to enable early de-risking of inducible neurotoxicity

Drug development is extremely costly and challenging [24]; moreover, lack of confidence in translatability often leads to failure during clinical trials. Neurotoxicity caused by candidate

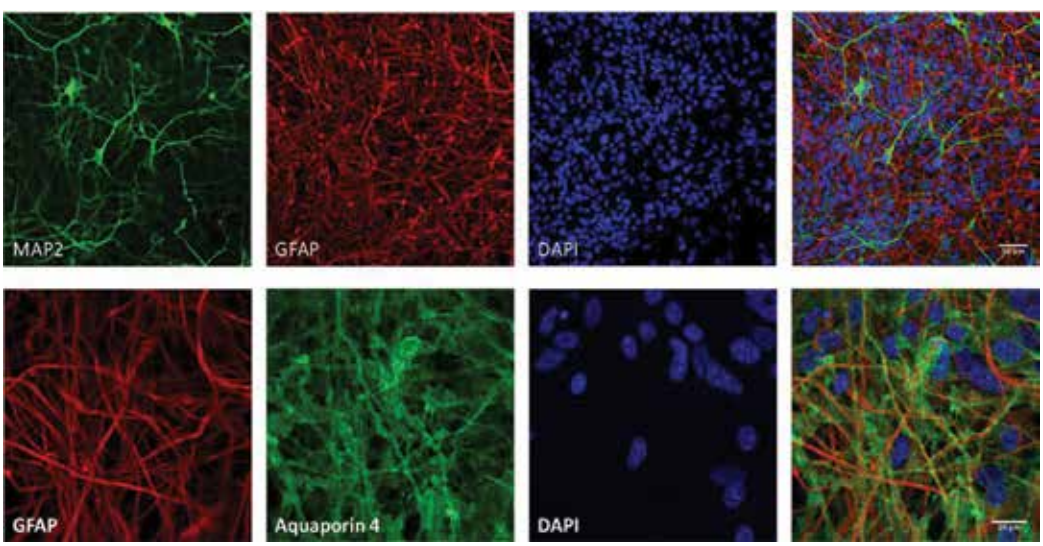

Figure 8. StemoniX's 2D human MicroBrain® model is comprised of human iPS cells-derived cortical neurons and astrocytes that are amendable to glutamatergic/GABAergic modulation. This model shows typical identity and functional markers, including MAP2: Neuronal marker; GFAP: Astrocyte marker; Synapsin I: Neuronal functional (synapse) marker; Aquaporin4: Astrocyte functional marker and DAPI: Nuclear marker [29–30].

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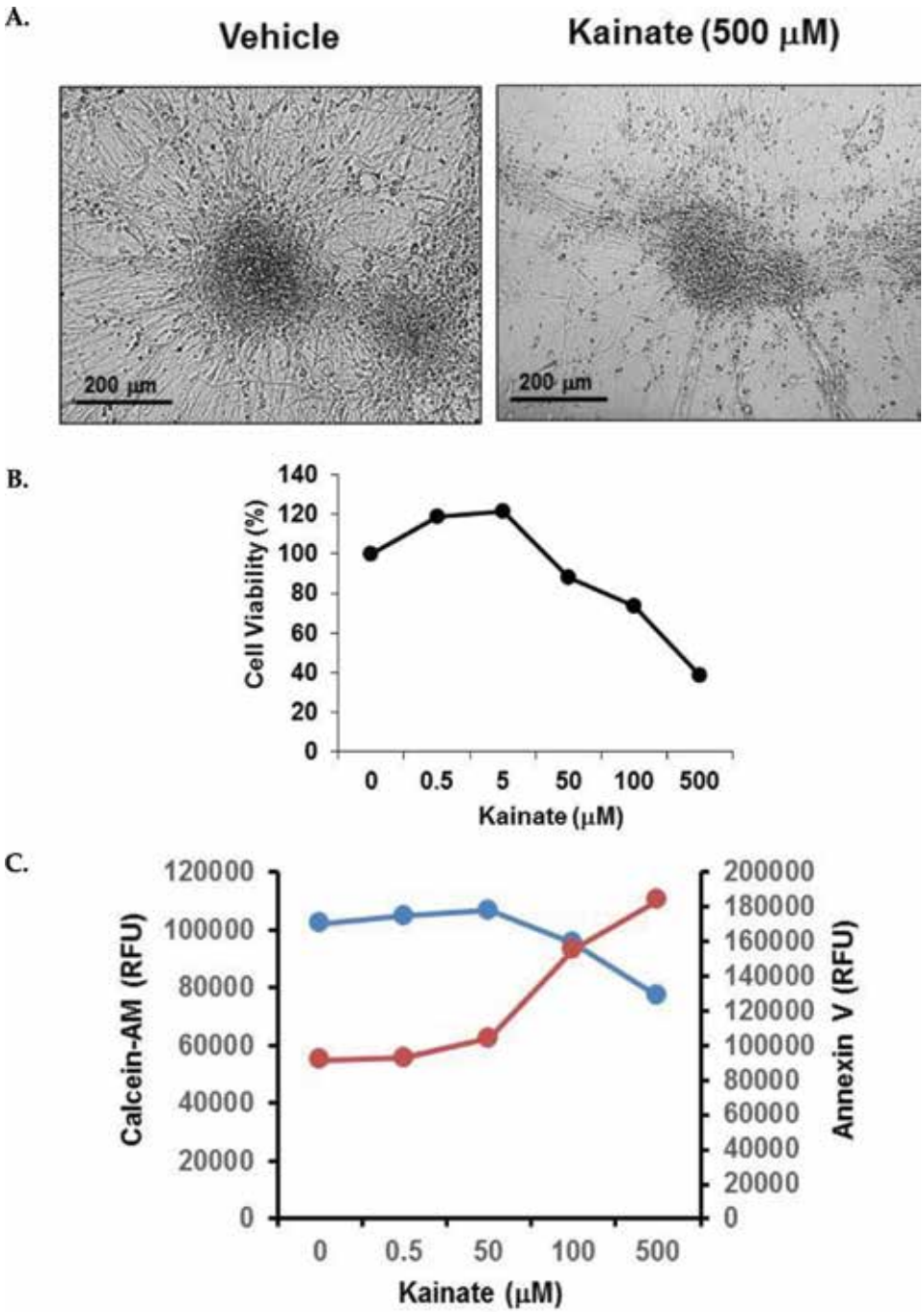

Figure 9. Changes in cell density of StemoniX's pre-plated, assay ready high throughput 2D human glutamatergic MicroBrain® platform (DIV33) imaged in bright field using an EVOS XL digital inverted microscope (A). Quantitative reductions in cell density were observed following 24-h exposure to kainate when compared to vehicle (1% DMSO) using a CellTiter-Glo® Luminescent Cell Viability Assay kit (B). Calcein-AM (blue line) and Annexin V (red line) assays were used to detect kainate-induced loss of cell viability and increased apoptosis, respectively (C).

drugs can lead to temporary or permanent harm to the central or peripheral nervous system. In case of glutamate excitotoxicity, excessive stimulation of the neurons occurs due to brain injury. Therefore, a major goal of the pharmaceutical industry has been to reduce late stage compound attrition due to neurotoxicity. Pharmaceutical companies, consortia (the Health and Environmental Sciences Institute) and regulatory authorities continue to establish more predicative models and biomarkers for early identification of neurotoxicity [25]. It is generally accepted, that iPSC-derived GlutaNeurons offer advantages over *in vivo* models: (1) isolated cells for glutamatergic signal transduction assessment in a dish; (2) low- and/or high-throughput screening at less cost and time than *in vivo* study conduct, although the MEA data interpretations may take several weeks; and (3) mechanistic assessment of neurotoxicity *in vitro* [26, 27]. Considering that candidate drugs may exhibit deleterious excitotoxic liabilities, iPSC derived GlutaNeurons support screening tools for early de-risking potentially translatable neurotoxicity. iCell® GlutaNeurons provide a relevant, excitatory neuronal model that enables researchers to study human neuronal network development and activity through interrogation and manipulation of relevant pathological pathways, thereby providing a new and valuable tool for drug discovery.

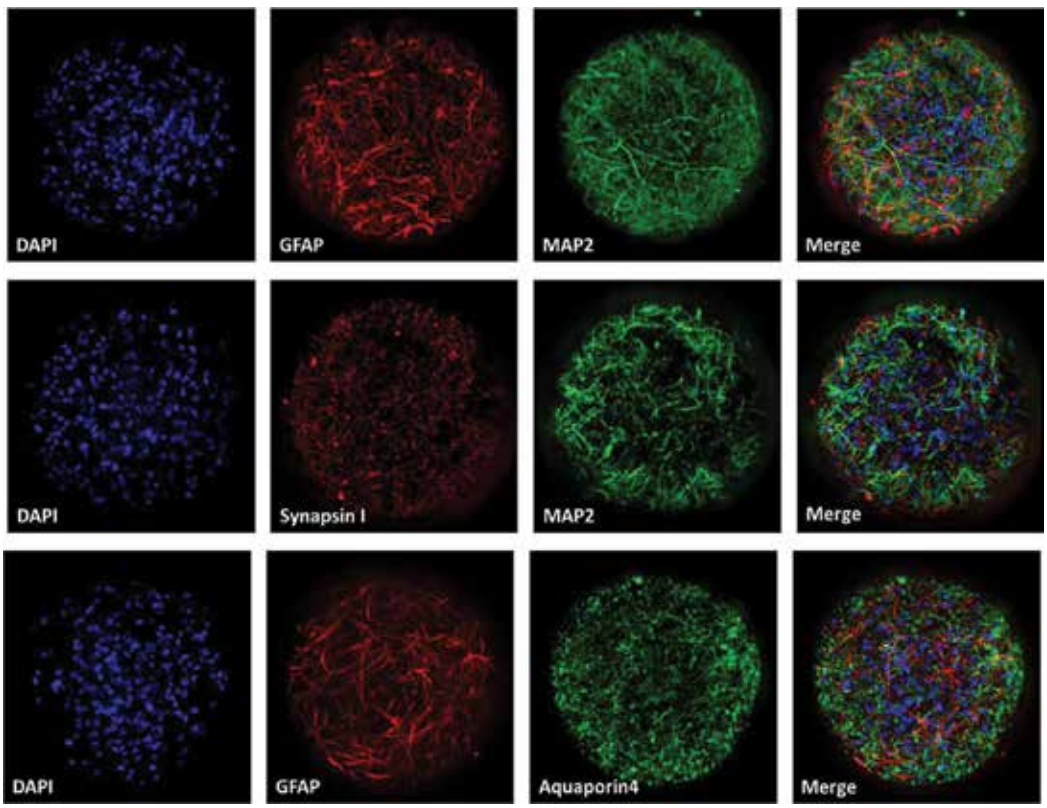

Figure 10. The StemoniX 3D Human MicroBrain® model contains a balanced population of cortical neurons and astrocytes which display typical identity and functional markers, including MAP2: Neuronal marker; GFAP: Astrocyte marker; Synapsin I: Neuronal functional (synapse) marker; Aquaporin4: Astrocyte functional marker and DAPI: Nuclear marker [30].

Previous studies revealed changes in brain organoid viability, proliferation, differentiation, and migration [28]. Newer human neural models try to replicate the brain complexity incorporating key features of the organ, such as the presence of a functional population of astrocytes. The MicroBrain® 2D model from StemoniX, (**Figure 8**) consists of a physiological relevant mixture of human iPS cells-derived cortical neurons and astrocytes [29]. Tests using this system have the potential of better recapitulate the intricate interplay between different neural cells and physiological response observed *in vivo* (**Figure 9**).

Recently, 3D human brain organoids have been used to assess spontaneous and synchronized neuronal activity and glutamate excitotoxicity. One interesting model is that the MicroBrain 3D Assay Ready Plates® (StemoniX), which consists of human cortical neurons and astrocytes spheroids (**Figure 10**) in 384-well format [29], containing one spheroid per well. These neural brain organoids are highly homogenous in size at approximately 500 μm and show phenotypic markers of mature cellular (e.g., synaptic proteins and glutamate transporters). Quantifiable synchronized spontaneous calcium oscillations may be detected from this 3D MicroBrain platform using a kinetic, high-throughput Fluorometric Imaging Plate Reader (FLIPR®). High speed confocal imaging confirmed homogenous calcium oscillations at the cellular level, whereas MEA analysis demonstrated robust synchronous glutamatergic/GABAergic circuitry. Moreover, synchronized calcium oscillations may be utilized as a biomarker for neuronal network activity in 3D MicroBrains. Finally, the MicroBrain 3D platforms are offered as preplated cells delivered at room temperature, speeding up the turnover of results, dispensing the need for long-term culture in the lab before the assay.

7. Recent advances in the development and validation sensitive and specific biomarkers for drug-induced neurotoxicity using iPSC-derived neuronal models

As noted above, pharmaceutical companies, the Health and Environmental Sciences Institute consortia and regulatory authorities have committed resources intended for developing and validating sensitive and specific biomarkers for early detection of drug-induced neurotoxicity [25]. Recent studies have shown synchronized calcium oscillations as a useful biomarker for neuronal network activity in CDI's 2D GlutaNeurons using the Hamamatsu FDSS7000EX/μCELL (a camera-based kinetic plate reader) [31] (**Figure 11**) and Stemonix's 3D MicroBrains using FLIPR [30] when cultured in 384-well plates (**Figure 12**). Synchronous spontaneous (MicroBrain 3D) or 4-AP-induced (GlutaNeurons) calcium flux are evident in these *in vitro* platforms. Taken together, inducible calcium flux in these models appears as a value-added parameter to enable early discovery compound screening. Additional studies will reveal the variables between *in vivo* calcium changes in the brain and those noted using various neuronal on-a-dish models as well as the translational values for calcium flux to MEA data, when monitoring for changes in electrophysiological parameters related to neurotoxin exposures.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate post-transcriptional gene expression in pathologic processes and can be found in plasma, serum and brain tissue, for example, circulating miRNA concentrations were differentially expressed in epilepsy patients

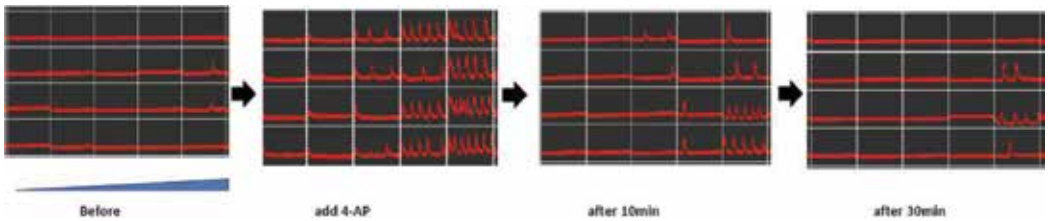

Figure 11. Representative detection of 4-aminopyridine (4-AP)-induced synchronization of calcium oscillations in CDI's 2D GlutaNeurons at DIV28 (post seeding). Interestingly, spontaneous basal synchronized calcium flux activities are not evident in CDI's 2D GlutaNeurons. N = 4 replicates. 5-point dose concentration response condition. Each well was normalized to concurrent control.

compared healthy controls [32]. Notably, significantly decreased expression level changes in serum hsa-miR-15a-5p showed sensitivity/specificity for diagnosis of epilepsy. To determine the utility of miRNAs in a rat model of acute experimental seizure, we evaluated miRNA expression level changes in serum as well as flash frozen cerebrum (cortex) and hippocampus from male Sprague Dawley rats that received the prototype compound, pentylenetetrazol, PTZ by single I.P. injection (**Figure 13**). In this study, single injection of PTZ at 60 mg/kg, I.P. was a subconvulsive dose, while single injection of PTZ at 80 mg/kg, I.P. induced decreased activity, straub tail, whole body tremor, heavy breathing, decreased body temperature (cold to touch), and seizures (irregular clonus of head and fore and hind limbs) by approximately 20 min post dosing. Serum glucose (a conventional seizure biomarker) was increased at the time of acute convulsive activity. Using the Abcam Fireplex™ Multiplex Rodent Discovery miRNA Toxicity Panel, significantly upregulated (35) or downregulated (26) miRNA expression level changes were detected from PTZ-treated rats with seizures versus controls without seizures; cerebral cortex revealed that miRNAs were significantly upregulated (1) or downregulated (5) in the PTZ-treated rats versus controls; and hippocampus revealed that miRNAs were significantly upregulated (8) or downregulated (3) in the PTZ-treated rats versus controls (data not shown). The miRNA profiles differed between cerebral cortex and hippocampus. As have been report for epilepsy patients, PTZ-induced acute seizures in rats were characterized by significantly decreased expression level changes in miR-15a-5p in cerebral cortex and serum (**Figure 12**). Taken together, circulating miR-15a-5p could be a minimally-invasive biomarker to enable monitoring for epilepsy in human patients [32] and PTZ-induced rat models of acute seizure. Additionally, retrospective analysis revealed 6 significantly upregulated miRNAs (mmu-mir-664-3p, mmu-mir-137-3p, mmu-mir-466i-5p, mmu-mir-204-5p, mmu-mir-221-3p, mmu-mir-29c-5p) and 3 significantly downregulated miRNAs (mmu-mir 324 5p, mmu-mir-140-5p, mmu-mir-328-3p) in male SD rats with seizures following once daily injections of the KA (8 mg/kg/day, i.p.) for 7 consecutive days versus control (vehicle: saline-treated) rats without seizures (author's unpublished data). These miRNAs showed promise as tissue-based exploratory biomarkers in rats with brain injury following KA-induced experimental seizures. Additionally, early data suggest that selected miRNAs detected from paraffin-embedded brain tissue slices may also correlate with profiles in hippocampal lesions (author's unpublished data).

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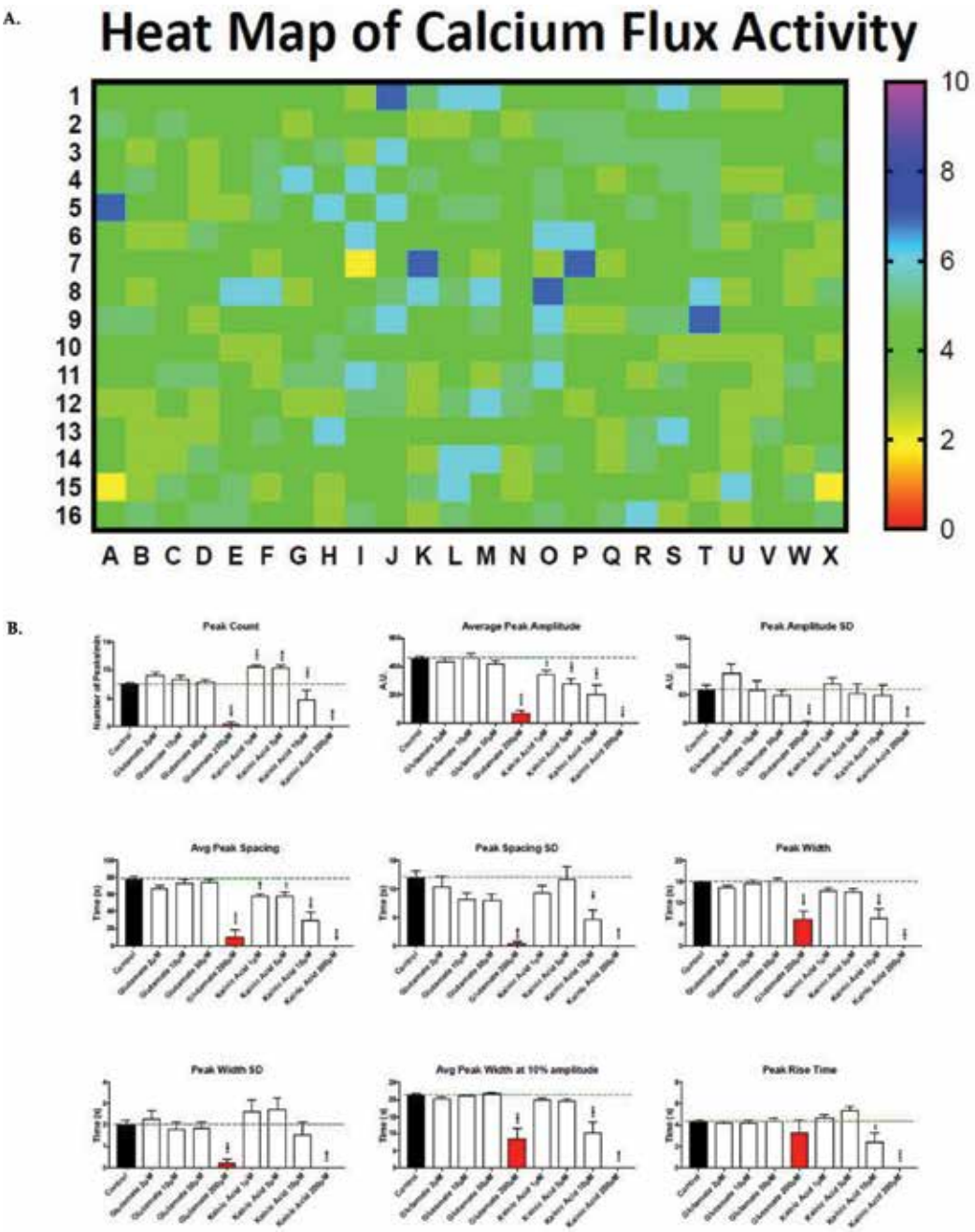

Figure 12. Representative heat map of spontaneous basal synchronized calcium flux activity in StemoniX's 3D Human MicroBrain® (A) and characterization of synchronous glutamatergic calcium flux (30 min) post glutamate, kainate or control treatment (B). N = 6 replicates. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (when compared to concurrent control).

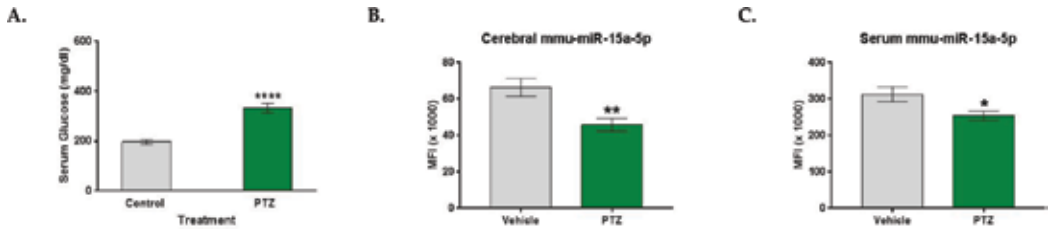

Figure 13. Male Sprague Dawley rats received a single I.P. injection of either vehicle (saline for injection USP, n = 10) or pentylenetetrazol (PTZ, 80 mg/kg, n = 10). In 10/10 PTZ-treated rats, decreased activity, straub tail, whole body tremor, heavy breathing, decreased body temperature (cold to touch), and seizures (irregular clonus of head and fore and hind limbs) were evident by approximately 20 min post dosing. Significant increases in serum glucose concentrations were detected in the PTZ-treated rats when compared to controls (A). Using the Abcam Fireplex™ Multiplex Rodent Discovery miRNA Toxicity Panel, PTZ-induced acute seizures in rats were characterized by significantly decreased expression level changes in miR-106b-5p in cerebral cortex (B) and serum (C). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (when compared to concurrent control).

When compared to those derived from animal models and/or animal-derived cell systems, miRNAs from iPSC-derived human neuronal models which may have greater potential for translation to human serum-based biomarkers. Interestingly, profiling of secreted miRNAs as novel biomarkers of neurodegeneration in CDI's iPSC-derived human neurons following acute (24 h) exposure to selected neurotoxicants has been reported [33]. Lessons learned from these studies included leveraging human iPSC-derived neurons at well-characterized stages of decline in morphology as indicated by high-content imaging analysis of neurite outgrowth parameters and cell viability (ATP release assay on replicate plates). Hence, CDI's human iPSC-derived neurons were treated at 2 h post-plating with 0.1–100 μM of neurotoxicants (including but not limited to bisindolylmaleimide I, colchicine, doxorubicin, paclitaxel and rotenone) or acetaminophen (negative control). Acute miRNA expression level changes were determined from conditioned cell culture medium, while adhered cells were analyzed using high-content imaging analysis. The low compounds concentrations had no/minimal effect on cell viability but with a significant decrease in neurite outgrowth, while the high compound concentrations induced significant decreases in both endpoints. No effect on any endpoint was observed with acetaminophen. Where relevant, neurotoxins that induced at least a 2-fold change in miRNA expression were potentially related to a decrease in neurite outgrowth, with further clustering based on association to cell viability, yielding: (1) no effect, (2) significantly decreased, or (3) independent of effect; indicating a relationship of miRNAs to progressive stages of neurodegeneration. While further investigations are warranted, the miRNAs profiles identified in cell culture medium of treated CDI's iPSC derived neurons may have greater translation to human serum-based biomarkers than those derived from either rodent cell lines and/or animal models of neurodegenerative disease(s). The usefulness of monitoring changes in miRNA profiles from 3D iPSC-derived neuronal models (e.g., Stemonix's 3D MicroBrains) has not been reported.

8. Summary

In vitro models support risk assessment in drug discovery and development. While, the extrapolation of toxicity data from animal models to humans is imperfect due to differences in anatomy and physiology, these traditional models are used because they represent whole-organism

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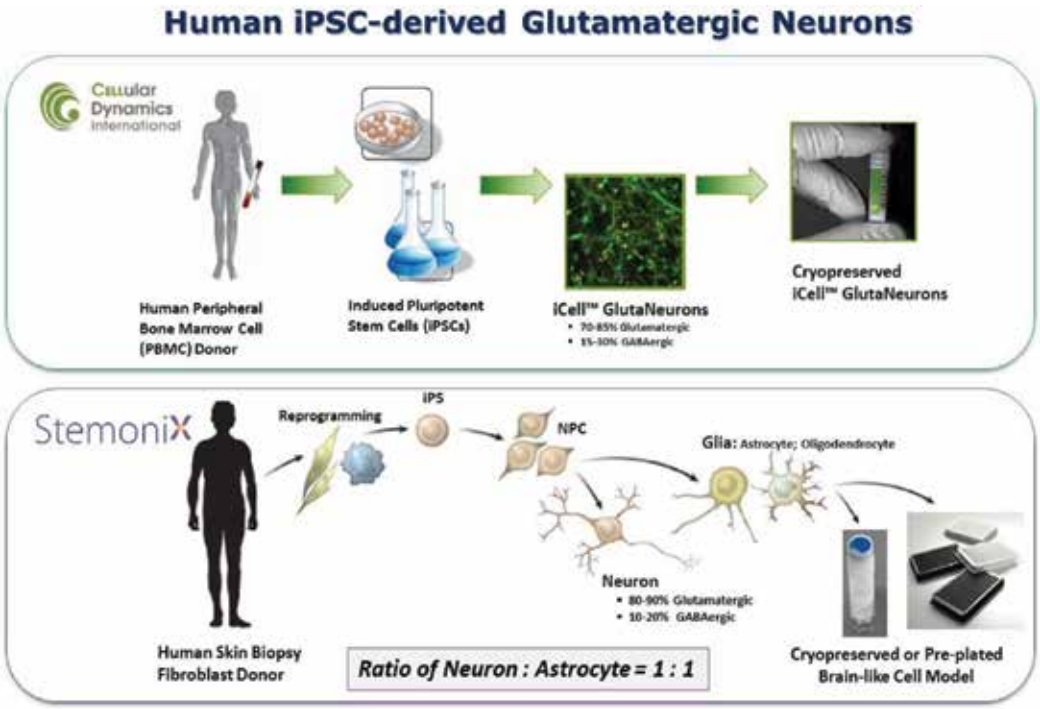

Figure 14. Adapted cartoons of the Human iCell® GlutaNeurons (Cellular Dynamics International) and Human MicroBrain® model which contains a balanced co-culture of cortical neurons and astrocytes (StemoniX).

biology that is not well replicated by *in vitro* methods. Increasing pressure to Replace, Reduce, and Refine the use of animal models in toxicity testing has propelled the use of *in vitro* systems to characterize the cellular and molecular mechanisms underlying biological changes associated with neurotoxicant exposure. The availability of iPSC-derived human GlutaNeuron cell models (**Figure 14**) provides relatively easy access to previously unattainable cell types from the human CNS system, which not only offer a unique source of neuronal cell types for candidate drug screening, but also provide new platforms to enable addressing the limitations of conventional animal models and primary cell cultures commonly used for mechanistic assessment of potentially translatable inducible excitotoxicity in drug discovery.

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at Hamamatsu for the courtesy image (**Figure 11**). I would also like to thank Sinae Lee, Ph.D. for her assistance regarding experimentation and imaging for the 2D iPSC-derived human glutamatergic MicroBrain® platform (**Figures 1**, **5** and **9**). Finally, I'd like to thank Dr. Jennifer Cohen at Takeda for lessons learned from studying miRNAs in CDI's iPSC-derived human neuronal model.

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References

[1] El-Ansary A, Al-Salem HS, Asma A, Al-Dbass A. Glutamate excitotoxicity induced by orally administered propionic acid, a short chain fatty acid can be ameliorated by bee pollen. Lipids in Health and Disease. 2017;**16**:96-104. DOI: 10.1186/s12944-017-0485-7

[2] Augustine GJ. Illuminating the location of brain glutamate receptors. Nature Neuroscience. 2001;**4**:1051-1052. DOI: 10.1038/nn1101-1051

[3] Gordon J, Amini S, White M. General overview of neuronal cell culture. Methods in Molecular Biology. 2014;**1078**:1-8. DOI: 10.1007/978-1-62703-640-5_1

[4] Prajumwongs P, Oratai Weeranantanapan O, Jaroonwitchawan T, Noisa P. Human embryonic stem cells: A model for the study of neuronal development and neuronal diseases. Stem Cells International. 2016;**1078**:1-9. DOI: 10.1155/2016/2958210

[5] Xu X-h, Zhong Z. Disease modeling and drug screening for neurological diseases using human induced pluripotent stem cells. Acta Pharmacologica Sinica. 2013;**34**:755-764. DOI: 10.1038/aps.2013.63

[6] Zeng H, Guo M, Martins-Taylor K, Wang X, Zhang Z, Park JW, Zhan S, Kronenberg MS, Lichtler A, Liu HX, et al. Specification of region-specific neurons including forebrain glutamatergic neurons from human induced pluripotent stem cells. PLoS One. 2010;**5**:1-7. DOI: 10.1371/journal.pone.0011853

[7] D'Aiuto et al. Large-scale generation of human iPSC-derived neural stem cells/early neural progenitor cells and their neuronal differentiation. Organogenesis. 2014;**10**(4):365-377. DOI: 10.1080/15476278.2015.1011921

[8] Sakore S, Chakraborty B. In vitro–in vivo correlation (IVIVC): A strategic tool in drug development. Journal of Bioequivalence & Bioavailability. 2011;**S3**:1-12. DOI: 10.4172/jbb.S3_001

[9] Vaarmann A, S Kovac S, Holmström KM, Gandhi1 S, Abramov AY. Dopamine protects neurons against glutamate-induced excitotoxicity. Cell Death & Disease. 2013;**4**:e455. DOI: 10.1038/cddis.2012.194

[10] Chiechio S. Chapter three—Modulation of chronic pain by metabotropic glutamate receptors. Advances in Pharmacology. 2016;**75**:63-89. DOI: 10.1016/bs.apha.2015.11.001

[11] Institute of Medicine (US) Forum on Neuroscience and Nervous System Disorders. Glutamate-Related Biomarkers in Drug Development for Disorders of the Nervous System: Workshop Summary. Overview of the Glutamatergic System. Washington (DC): National Academies Press (US); 2011. 2. Available from: https://www.ncbi.nlm.nih.gov/books/NBK62187/

[12] Obata K. Synaptic inhibition and γ-aminobutyric acid in the mammalian central nervous system. Otsuka M, editors. Proceedings of the Japan Academy. Series B, Physical and Biological Sciences. 2013;**89**(4):139-156. DOI: 10.2183/pjab.89.139

[13] Kim K, Lee S-G, Kegelman TP, et al. Role of excitatory amino acid transporter-2 (EAAT2) and glutamate in neurodegeneration: Opportunities for developing novel therapeutics. Journal of Cellular Physiology. 2011;**226**(10):2484-2493. DOI: 10.1002/jcp.22609

[14] Zhou Y, Danbolt NC. Glutamate as a neurotransmitter in the healthy brain. Journal of Neural Transmission. 2014;**121**(8):799-817. DOI: 10.1007/s00702-014-1180-8

[15] Sonnewald U, Schousboe A. Introduction to the glutamate–glutamine cycle. In: Schousboe A, Sonnewald U, editors. The Glutamate/GABA-Glutamine Cycle. Advances in Neurobiology. Vol. 13. Cham: Springer; 2016. DOI: 10.1007/978-3-319-45096-4 1

[16] Ruiz A, Matute C, Alberdi E. Intracellular Ca^{2+} release through ryanodine receptors contributes to AMPA receptor-mediated mitochondrial dysfunction and ER stress in oligodendrocytes. Cell Death & Disease. 2010;**1**(7):e54. DOI: 10.1038/cddis.2010.31

[17] KD1 B, Edwards TM, Rickard NS. The role of intracellular calcium stores in synaptic plasticity and memory consolidation. Neuroscience and Biobehavioral Reviews. 2013 Aug;**37**(7):1211-1239. DOI: 10.1016/j.neubiorev.2013.04.011

[18] Gorman AM. Neuronal cell death in neurodegenerative diseases: Recurring themes around protein handling. Journal of Cellular and Molecular Medicine. 2008;**12**(6a):2263-2280. DOI: 10.1111/j.1582-4934.2008.00402.x

[19] Dolmetsch R, Geschwind DH. The human brain in a dish: The promise of iPSC-derived neurons. Cell. 2011;**145**(6):831-834. DOI: 10.1016/j.cell.2011.05.034

[20] Young JE, Goldstein LSB. Alzheimer's disease in a dish: Promises and challenges of human stem cell models. Human Molecular Genetics. 2012;**21**(R1):R82-R89. DOI: 10.1093/hmg/dds319

[21] Fujikawa DG. The role of Excitotoxic programmed necrosis in acute brain injury. Computational and Structural Biotechnology Journal. 2015;**13**:212-221. DOI: 10.1016/j.csbj.2015.03.004

[22] McConnell ER, McClain MA, Ross J, LeFew WR, Shaferb TJ. Evaluation of multi-well microelectrode arrays for neurotoxicity screening using a chemical training set. Neurotoxicology. 2012 Oct;**33**(5):1048-1057. DOI: 10.1016/j.neuro.2012.05.001

[23] Kasteel EEJ, Westerink RHS. Comparison of the acute inhibitory effects of Tetrodotoxin (TTX) in rat and human neuronal networks for risk assessment purposes. Toxicology Letters. 2017;**270**:12-16

[24] Cao S-Y, Hu Y, Cheng C, Yuan F, Xu M, Li Q, Fang K-H, Chen Y, Liu Y. Enhanced derivation of human pluripotent stem cell-derived cortical glutamatergic neurons by a small molecule. Scientific Reports. 2017;**7**:3282-3292

[25] Roberts RA, Aschner M, Calligaro D, Guilarte TR, Hanig JP, Herr DW, Hudzik TJ, Jeromin A, Kallman MJ, Liachenko S, Lynch JJ 3rd, Miller DB, Moser VC, O'Callaghan JP, Slikker W Jr, Paule MG. Translational biomarkers of neurotoxicity: A Health and Environmental Sciences Institute perspective on the way forward. Toxicological Sciences. 2015 Dec;**148**(2): 332-340. DOI: 10.1093/toxsci/kfv188

[26] Choi DW. Glutamate neurotoxicity and diseases of the nervous system. Neuron. 1988;**1**: 623-634

[27] Mattson MP. Glutamate and neurotrophic factors in neuronal plasticity and disease. Annals of the New York Academy of Sciences. 2008;**1144**:97-112. DOI: 10.1196/annals.1418.005

[28] Lee C-T, Bendriem RM, Wu WW, Shen R-F. 3D brain organoids derived from pluripotent stem cells: Promising experimental models for brain development and neurodegenerative disorders. Journal of Biomedical Science. 2017;**24**:59. DOI: 10.1186/s12929-017-0362-8

[29] McDuffie JE. Microphysiological models informing target assessment to translational biomarkers. Invited Presentation at: 3D Tissue Models 2017 Conference. Boston, MA, USA; August 2017. http://3d-tissuemodels.com/about/speakers-2/

[30] Dea S, Biesmans S, Mora-Castilla S, Romero S, Saleh A, Zanella F, Carromeu C. Microphysiological models informing target assessment to translational biomarkers. Poster Presented at: 3D Tissue Models 2017 Conference. Boston, MA, USA; August 2017. http://3d-tissuemodels.com/about/about-event/

[31] Hisada S, Shouming D, Kato N, Matsubara M, Iwase F. High throughput and high-speed data acquisition of compounds evaluation on calcium oscillation of human iPSC derived cortical neurons. 2017 Society of Neuroscience Meeting. San Francisco, CA, USA; November 2017. https://www.sfn.org/annual-meeting/neuroscience-2017

[32] Wang J, Tan L, Tan L, Tian Y, Ma J, Tan C-C, Wang H-F, Liu Y, Tan M-S, Jiang T, Yu J-T. Circulating microRNAs are promising novel biomarkers for drug-resistant epilepsy. Scientific Reports. 2015;**5**:10201-10210. DOI:10.1038/srep10201

[33] Cohen JC, Nagatome K, Tanaka Y, Uchiyama H. Profiling secreted miRNA as biomarkers of neurodegeneration in human iPSC-derived neurons. Poster 453 Presented at: Society of Toxicology Annual Meeting, Balimore, MD, USA, March 2017. https://cellulardynamics.com/event/sot-56th-annual-meeting-toxexpo/

Chapter 3

Use of the Model Organism *Caenorhabditis elegans* to Elucidate Neurotoxic and Behavioral Effects of Commercial Fungicides

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Additional information is available at the end of the chapter

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Abstract

Fungicides are widely used in agriculture and medicine and there are several different types of fungicides that are distributed globally in the soil and water through water run-off and drift from spraying as well as other avenues of distribution. Understanding the biological effects of fungicide contaminants in the environment on non-target organisms including humans is critical. This chapter considers the use of the model organism and key beneficial soil nematode, *Caenorhabditis elegans*, as an effective strategy for examining fungicide effects on growth, reproduction, nervous system and behavior. We present novel evidence of the effects of a general use fungicide, mancozeb, on behavior and neuronal structure.

Keywords: mancozeb, fungicide, dithiocarbamate, *C. elegans*, neurotoxicity, dopamine, neurodegenerative

1. Introduction

Fungal infestations of agriculturally important crops, like the infamous potato late blight (*Phytophthora infestans*) that caused widespread famine and death in the 1800s in Ireland, are estimated to be responsible for at least 13% of crop losses in the world annually. The *Magnaporthe grisea* fungus, an Ascomycota, is considered one of the most damaging in the world, infesting rice crops at a devastating annual loss [1]. In addition to agricultural blights, rusts, smuts, mildews and other types of fungal infections, fungal infestations are responsible for the Chestnut blight (*Cryphonectria parasitica*) that caused the near annihilation

of the American Chestnut tree and Dutch elm disease, caused by two different fungi carried by the elm bark beetle, that is currently decimating American elm trees. In addition to plant fungal diseases, pathogenic fungi infect many different animals, from beneficial insects to humans. For example, *Ascosphaera apis* is a fungus that devours honey bee larvae and Stonebrood is a fungal infection that affects honey bees, as well as other insects, mammals and birds [2]. Further, animals can unwittingly participate in the fungal lifecycle and so serve as reservoirs of pathogenic fungi. Many fungal infections in humans are opportunistic, like *Candida albicans* (yeast infections) and Athlete's foot (*Trichophyton mentagrophytes*) [1]. Fungal infections are often highly contagious and resist treatment and so are of major concern to public health.

Fungicides are thus used both in the home and in agriculture. Dithiocarbamates like maneb, mancozeb and thiram were introduced commercially in the mid-twentieth century and many remain popular today. Multiple classes of organic fungicides are in current use for their ease of preparation, enhanced fungal toxicity, and reduced phytotoxicity. Currently, more than 80% of the agricultural sites in the US have regular treatment with fungicides [1], the majority of them are broad spectrum with multiple modes of action. Because many fungal infestations are difficult to eradicate, use of fungicides prophylactically in agriculture is common practice. In recent years, the use of fungicides to ward off fungal infections and to increase crop yields has soared, spurred in part by the increased production of a variety of fungicides. Fungicides are routinely and frequently applied to up to 30% of the US crops of soybean, corn and wheat. A recent study revealed that 75% of surface waters tested in the US and 58% of ground water contained measurable levels of fungicides known to be toxic to aquatic animals [3].

Understanding the biological effects of fungicides and fungicide mixtures on non-target organisms, including humans, is critical. While application levels of most fungicides seem to cause little overt toxicity in mammals, much lower doses have been linked to obesity in mice [4] and Parkinson's like symptoms in mammals, including humans [5]. In addition, early exposure to a number of fungicides has been linked to epigenetic, transgenerational occurrence of obesity in mammals [5]. Because fungicide contamination is found worldwide and these chemicals are heavily used, there is an urgent need to examine the effects of fungicides on growth, development and health of organisms that are exposed, including aquatic organisms, soil organisms, birds and mammals. This chapter will review current use of several fungicides and will argue that the use of the model organism, *Caenorhabditis elegans*, is an important strategy for examining the effects of fungicides on behavior, nervous system, growth and development.

2. Uses and variety of fungicides

A large and increasing number of fungicides are used for agriculturally critical crops like soybean, rice, corn and wheat, as well as for orchard fruits and vegetables. **Table 1** lists the most commonly applied chemical categories of fungicides for commercial agricultural use and their major mode for action [6]. Many fungicides are applied topically to either the seed or the

Category of fungicide	Specific example	Major effect
Azole	Myclobutanil, fluconazole, flutriafol	Inhibit sterol synthesis
Pyrimidines	Fenarimol	Disrupt sterol synthesis
Strobilurins	Azoxystrobin	Inhibit mitochondrial respiration
Polyoxins	Polyoxin D	*
Benzimidazoles	Thiophanate-methyl	Inhibit cell division
Dicarboxamides	Vinclozolin	Inhibition of androgen signaling
Carbamates	Propamocarb	*Non-cholinergic mechanism
Dithiocarbamates	Mancozeb, thiram	Inhibit mitochondrial respiration
Aromatic hydrocarbons	Chloroneb	Uncouple oxidative phosphorylation
biofungicides	Soilguard	Microorganism defense
Carboxamides	Boscalid	Inhibit succinic acid oxidation

*Unknown mechanism of toxicity.

Table 1. Classes of commonly applied fungicides and major mode of action (adapted from [6]).

plants and the ground surrounding and act to repel fungal spores and prevent fungal growth and infection. These are considered less toxic to humans, except for workers involved in their application. Contact fungicides include the dithiocarbamates and aromatic hydrocarbons [6]. These compounds wash off and can enter the water supplies and intercalate into the soil. Acute neurotoxicity has been reported for the Mn^{2+}-containing ethylene-bis-dithiocarbamate fungicides mancozeb [7] and thiram [8] via selective dopaminergic and gabanergic neuron signaling associated with perturbations in mitochondrial respiration. Other fungicides can be absorbed into plant tissues, with either limited penetrance (strobilurins, dicarboxyamides) or systemic distribution (phosphonates). Vinclozolin is a dicarboximide fungicide that has antiandrogen activity in developing male brain [9] and is used primarily to treat diseases caused by *Botrytis cinerea* and *Monilinia* spp. [10]. In mammalian brain, the preoptic/anterior hypothalamic area (POA/AH) is one of the most sexually dimorphic areas as reported previously for male and female rabbit offspring with sexual dimorphism and endocrine disruption induced by vinclozolin. A previous report noted that secondary products of vinclozolin activity may result in lipid peroxidation as well as oxygen free radical turnover in *B. cinerea*. Further investigations are warranted to reveal whether metabolites of vinclozolin induced signaling mechanisms that lead lipid peroxidation and/or oxygen free radicals associated with neurotoxicity. Another carbamate fungicide, propamocarb, induced decreased motor activity and vacuolization of the choroid plexus in rats and interference with glycolysis and formation of fibrillary proteins like neurofilaments through interaction with SH-groups [11]. Fungicides are also applied post-harvest to reduce rot during food storage and transport, and residues persist after food processing. A few specific examples are discussed below. A comprehensive review of all the major types of fungicides is beyond the scope of this review and the reader is directed to several recent reviews [6, 12].

2.1. Azole fungicides

In general, azole fungicides inhibit the formation of the fungal cell wall by interfering with a key enzyme involved in ergosterol biosynthesis [13]. There are a large number of different azole fungicides, used both agriculturally and in medicine. Despite their widespread use, relatively little work has been done to examine the toxicity of these compounds to wildlife and humans. One commonly used azole, flutriafol, has been associated with liver toxicity, reduced body weight of offspring and possible endocrine disruption of female fertility in rats [13, 14]. Another azole, triadimefon, disrupts dopamine neurotransmission function in rats, with subsequent effects on the nigrostriatal system that governs behaviors like general locomotor activity, as well as stereotyped, repetitive behaviors [15]. One recent study demonstrated edema and necrosis of circular and longitudinal muscle layers in earthworms exposed to tebuconazole for 14 days [16]. Further, epoxiconazole, a commonly applied triazole, acts as a male reproductive toxicant in rats [17] and damaged spermatogenesis in *C. elegans* [18]. Pursuing studies using *C. elegans* is a very useful and important avenue of research.

2.2. Strobilurins

This class of fungicide, developed in the 1990s has rapidly become the second most prevalent type of fungicide, accounting for a $600 million industry that includes treatment of soybean, wheat and other cereal crops. Compounds like kresoxim-methyl and pyraclostrobin are broad-spectrum fungicides that have long-term stability [19] and were recently shown to be neurotoxic to cultured mammalian neurons [20]. Pyraclostrobin fed to rodents was also associated with stomach ulcerations, reduced weight gain and decreased food consumption [21]. In contrast, azoxystrobin did not cause significant toxicological health risks to humans from levels measured in the environment [22]. These compounds, while heavily used, have not been studied extensively by researchers, so much more work is needed.

2.3. Dithiocarbamates

Dithiocarbamate fungicides have been in use globally since the 1940s and are often considered to be only mildly toxic, or a moderate irritant, to mammals and human agricultural workers [23]. However, numerous reports have linked the exposure to these compounds to neurodegenerative damage to dopaminergic systems, including Parkinson's disease ([5, 24]; reviewed in Ref. [25]). Because many of these compounds chelate metal ions, particularly divalent cations like manganese, magnesium and zinc, the commercial formulations often contain these metal ions [26], which can exert toxic effects independently and in conjunction with the organic moieties in the formulations [27]. These compounds, including maneb, mancozeb and manate, have been shown to alter ion channel function [28] and inhibit mitochondrial function [29], as well as generate ROS (reactive oxygen species) [30]. The subsequent sections of this chapter focus on this fungicide as an example of the utility of *C. elegans* for examining fungicide effects on nervous system and behavior.

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3. Use of *C. elegans* as a model to assess fungicide effects on development, lifespan and the nervous system

A number of pesticide and fungicide toxicology studies utilize the earthworm (e.g., *Eisenia fetida*) because of the importance of earthworms in soil health [16]. However, this organism lacks the ease of cultivation and powerful genetics of the model organism, *C. elegans*. The soil nematode, *C. elegans*, is an excellent model organism for neurotoxicology study of fungicides [31]. *C. elegans* is an important mesofaunal soil nematode that consumes bacteria and is predated by fungi, other nematodes and a host of other soil organisms [32]. *C. elegans* is a free-living, non-parasitic nematode that grows from egg to adult in about 3.5 days at 20°C, with a lifespan of about 18 days at that temperature. The nematode is transparent, 1 mm in length as an adult and is easily grown on agar plates with small patches of *Escherichia coli* as food. Hermaphroditic with the possibility of sexual mating with a low frequency male phenotype, the nematodes each produce 300 progeny through self-fertilization and up to 1000 progeny if mated with a male. The genome has been fully sequenced and a number of laboratories have generated a rich variety of mutants, including strains that express green fluorescent protein under different promoters. The GFP strains allow researchers to examine particular cell types or tissues using fluorescence microscopy. While a simple organism with only 302 neurons and a total of 959 cells, *C. elegans* nonetheless exhibits numerous behaviors and sensory functions, including associative and non-associative learning. It uses all the major neurotransmitters found in other invertebrates and shares at least 60% genes with mammals. *C. elegans* can self-fertilize or reproduce sexually, making genetic-level studies straightforward. Its short life span, transparent body and ease of cultivation have made *C. elegans* a key model for neurotoxicological study [33].

3.1. *C. elegans* dopamine neurons

Fungicides can have long-term effects on soil organisms, including nematodes [34]. Early reports of parkinsonism and other dopaminergic neuron-mediated neurological conditions in agricultural workers exposed to these compounds [35–37] generated interest in examining the unintended mechanisms by which these fungicides exert neurotoxicological effects. *C. elegans* is an ideal organism for experimentally examining the neurotoxicological effects of these fungicides on dopamine neurons and the behaviors they govern. *C. elegans* have four pairs of dopaminergic neurons, two pairs of CEP (cephalic sensilla) neurons, one pair of ADE (anterior deirids) neurons and one pair of PDE (posterior deirids) neurons [38]. These neurons modulate a variety of behavioral responses including foraging behavior, locomotion rate upon entering food, transition from swimming to crawling, egg-laying and defecation [39, 40]. The neurons are also large, readily identified in the light microscope and can be evaluated for neurodegeneration using established morphological criteria [33].

The CEP neurons reside anterior to the circumpharyngeal nerve ring in the head region of the animal and have ciliated processes that extend to the mouth/nose tip region (**Figure 3A**). The neurons

synthesize and release dopamine [41] as well as express acetylcholinesterase, a target of many pesticides. These neurons modulate a variety of behavioral responses including foraging behavior and locomotion rate upon entering food [39, 40]. They regulate a characteristic feeding behavior called basal slowing, wherein the animal detects the presence of bacteria via mechanosensation transduced by these neurons and slows its foraging locomotion. CEP neurons modulate feeding/search behavior by altering the overall activity of a 48 neuron circuit [42]. The ADE neurons, posterior to CEP neurons but still slightly anterior to the circumpharyngeal nerve ring, serve as mechanoreceptors that regulate the animal's response to nose touch and the presence of bacteria. Like the CEP neurons, they regulate the basal slowing response to food, and in addition, respond to harsher nose touch with a recoil response. These neurons also seem to participate in responses to nematode pheromones that regulate foraging and the recovery from dauer formation [43].

The PDE neurons, found in the posterior half of the nematode, also regulate the basal slowing response upon nematode entry to a lawn of bacterial food [39]. These dopaminergic neurons have also been found to regulate more complex behaviors and functions like locomotion, sensory perception, and learning [39, 44–48]. Because there are straightforward behavioral assays of dopaminergic neuron function, and because there are readily available strains of nematodes that express GFP in dopaminergic neurons, *C. elegans* serve as a powerful model organism for examining the effects of fungicides on dopaminergic systems.

A number of dithiocarbamate fungicides, including maneb, ziram and Mancozeb, have been evaluated using the *C. elegans* model system. Mancozeb is a commercial formulation of the main active ingredients ethylene-bis-dithiocarbamate, zinc, manganese, as well as other "inactive" compounds that are not published by the company (Bonide Products, Inc., NY). It is used in agricultural and residential garden settings to control a variety of fungal infections that infest ornamental plants and vegetables, like blossom end rot and downy mildew [49]. Mancozeb is currently approved for use in over 120 countries including the United States. It is used on over 70 different crops and protects against more than 400 different fungal infections [50]. Typically, even though it has limited solubility in water, Mancozeb is applied as a mixture with water to plants and the soil around them, where it forms a film on the plants and intercalates into the upper layers of soil. According to the application instructions, Mancozeb is most typically applied at a final concentration in the range of 0.5–1.5% (v/v). Mancozeb has an active half-life of 1–7 days depending on weather conditions. The targeted mechanism of action of Mancozeb is the disruption of key metabolic enzymes by altering sulfhydryl groups. There is also evidence that the metabolite, ethylenethiourea (ETU) might also have toxic effects. The organic moiety of Mancozeb is a potential estrogen disrupting chemical and suspected carcinogen [51]. Further, the manganese and zinc present in the formulation also cause toxicity via multiple mechanisms. For example, zinc modulates glutamate neurotransmission and interacts with numerous enzyme systems [52] and manganese can exacerbate dopamine neuron degeneration [53] possibly via enhanced oxidative stress [54]. Mancozeb was shown to be lethal to nematodes at concentrations experienced by agricultural workers [55] and sublethal doses led to degeneration of neurons, including dopamine neurons [55], induction of heat shock responses [55] and inhibition of larval growth [56, 57]. In many instances, the fungicide is applied repeatedly, so organisms are exposed multiple times and cumulatively over long periods of time. It is likely that many people are exposed to low levels of Mancozeb

remaining on unwashed fruits and vegetables. Farmers, pesticide sprayers and home gardeners are exposed to higher levels when they work with the formulations. Thus, while soil and aquatic organisms may be most vulnerable to the toxicity of sublethal concentrations of mancozeb, human populations are also at risk from exposure to concentrated formulations and from the accumulated effects of small doses from ingesting fruits and vegetables that have been in contact with the fungicide [25, 58].

We explored locomotory, mechanosensory, and egg-laying behaviors in response to a 24 h exposure to Mancozeb to better understand the impacts of sublethal amounts of this widely used fungicide on this important soil organism [33] on particular neuronal populations.

4. Materials and methods

4.1. *C. elegans* strains and maintenance

The following strains were obtained from the *C. elegans* Genetics Center: wild-type (N2); OH7547 ((otIs199)[*cat2*::GFP + rgef-1(R25B3.3)::dsRed + *rol-6*(su1006)]). *C. elegans* was maintained on NGM petri plates containing spots of *E. coli* (OP50) bacteria for food at 20°C [59]. We prepared small developmentally-synchronized cultures by transferring 10 gravid worms to NGM plates with *E. coli* and allowing them to lay 30–50 eggs for approximately 2 h. Resulting synchronized cultures were exposed to mancozeb as described in the following section.

4.2. Mancozeb (MZ) exposure

Mancozeb treatments were made as volume:volume percent solutions with water, as recommended by the manufacturer. 0.5–1.5% mimic the application dose to garden soils [33] and are within the LC50 of nematode sensitivity of 1% [33, 55]. Lower concentrations correspond to putative residual concentrations present beyond the garden or application site. The solutions were spread evenly using a sterile glass rod to coat the entire surface of the NGM agar plate. In some experiments, the Mancozeb was also combined with the *E. coli* mixtures, while in others the *E. coli* were spread onto the plate already coated with Mancozeb. We saw no differences in experimental outcome among these different exposure conditions. In the experiments reported in this study, the Mancozeb-treated plates were allowed to air-dry overnight at 20°C prior to the placement of nematodes. Young adult nematodes, just past the L4 transition, were exposed to different concentrations of Mancozeb for 1, 3, 6 or 24 h. Some nematodes were tested directly after exposure and some were given a comparable recovery time on plates lacking Mancozeb. For example, a 24 h exposure to Mancozeb was followed by a 24 h recovery period on untreated plates prior to behavioral or microscopic assessment. For all experiments, at least three separate replicates were performed.

4.3. Behavioral measurements

For each assay, unexposed, age-matched nematodes were measured during the same time frame as the Mancozeb-exposed nematodes. More than one investigator conducted the

behavioral measurements to prevent investigator bias. All measurements were obtained at room temperature (22°C). Nematodes were visualized with an Olympus SZ-1 dissecting microscope.

4.3.1. Basal slowing behavior

When nematodes enter a patch of *E. coli* food, they dramatically slow their locomotion, a behavior called basal slowing, whose mechanism involves the activation of the dopaminergic mechanosensory neurons, CEP, ADE and PDE [39]. After exposure to Mancozeb or control conditions, nematodes were transferred individually to plates lacking food for several seconds to allow the adhering Mancozeb to be cleared. Control nematodes also were transferred to food-free plates for this brief "clearing" period. Then, nematodes were transferred individually to petri plates containing a circular patch of *E. coli*. They were placed outside of the food circle. Basal slowing was measured by counting body bends for 20 s outside of the food and then again when the nematode entered the patch of food. Body bends were defined as one complete sinusoidal movement of the nematode's tail. We operationally defined basal slowing as the difference in body bends/20 s on the food as compared with off the food, as determined by earlier studies [60]. Basal slowing responses were compared with untreated animals using a one-way ANOVA followed by Bonferroni tests for multiple comparisons using Prism 5.0 statistical software (GraphPad, Inc.) for experiments testing more than one concentration of Mancozeb and the Student's *t*-test for the reversibility assay, as described in specific figure legends.

4.3.2. Tail drag (or paralysis) after swimming

Our earlier work determined that Mancozeb exposure interfered with overall locomotion frequency [33]. We further examined locomotion by developing a behavioral measure of tail motion. We noticed that, following exposure to liquid-induced swimming behavior, the return to crawling behavior was abnormal. In particular, the tail appeared stiffer or paralyzed. We measured this change in tail movement as a novel assay of locomotory deficit. Individually, worms were removed from the treatment plate and placed on a 2 cm petri dish lacking *E. coli*. The dish was placed on an Olympus SZ-1 dissecting microscope, and locomotion was digitized as short video clips using a digital camera (Insight, Inc.). 2 μl of M9 buffer solution were placed over the worm to induce swimming behavior, and left to dry (about 30 s). Directly after the M9 buffer solution had dried, we collected 100 frames (at 60 frames/s) of worm movement using Spot (2.0) software (Insight, Inc.). The worm was then removed from the dish, and a new worm was put in its place. This continued until all the worms from the Mancozeb-coated petri dish or from control conditions were filmed.

The computer program LoggerPro (Vernier, Inc.) tracked the movement of the worms from the short video clips. Two different data points marking the head and the tail were placed on each worm image for each frame of the video and the X and Y coordinates of the points were determined. We calculated the distance each worm traveled between frames (distance = sqrt($(X_2 - X_1)^2 + (Y_2 - Y_1)^2$)). This allowed the speed of the head and the tail to be

found for each worm at each time point (speed = $d/(t_2 - t_1)$). These values were then averaged for each worm. We focused our statistical analyses on the tail movements. The tail speeds were analyzed with ANOVA followed by Bonferroni's post-hoc tests for multiple comparisons to determine significance between different treatments. All statistical analyses were performed using Prism 5.0 software (GraphPad, Inc.). A p-value of less than 0.05 was considered statistically significant.

4.4. Confocal microscopy

Dopamine neurons were evaluated immediately after a 24 h exposure to Mancozeb or control conditions by immobilizing young adult hermaphrodite nematodes of the OH 7547 strain on a 2% agarose pad within a drop of buffer containing 2.5 mM NaN_3 to paralyze the worms. Images were acquired with a Nikon PCM2000 confocal laser-scanning microscope equipped with an argon laser. For each neuron analyzed, we collected 18-image z-stacks using a 60× immersion oil lens and analyzed the merged images. Confocal images were processed to adjust contrast using simple PCI software. In all cases, whole images were processed, rather than particular portions of images, to ensure faithful representation of the results.

5. Results and discussion

We discovered that sublethal concentrations of Mancozeb impaired locomotion, both exploratory locomotion and the transition from swimming to crawling behavior, a transition that is activated by dopamine neurons [33]. With further analysis using our tail drag assay, we documented a significant tail rigidity or paralysis upon return to crawling behavior (**Figure 1A**). The tail paralysis persisted even after 24 h recovery from the exposure to Mancozeb (**Figure 1B**). In addition, the basal slowing response of fungicide-exposed nematodes was significantly impaired (**Figure 2**). If nematodes were exposed to the fungicide for 24 h and allowed to recover for 24 h, the behavior did not recover, suggesting a long-lasting and potentially permanent impairment.

The locomotory transition from swimming to crawling, a process governed by dopamine neurons, was also permanently impaired [33]. In keeping with these long-term functional impairments, the dopaminergic neurons exhibited characteristic indications of neurodegeneration, including blebbing along the neural processes and changes in soma morphology (**Figure 3**) [33]. Other recent studies have also documented neuronal impairments in response to maneb and manganese exposure, measured by a loss of GFP fluorescence intensity [27, 55].

While dopaminergic neurons were most sensitive to fungicide exposure [33, 56], other neuron groups, including serotonergic [33], glutamatergic and GABA neurons [50, 61] exhibited morphological damage. Vulnerability to neurodegenerative effects varies depending on the dose of fungicide, duration of exposure and time of life of exposure [25, 33].

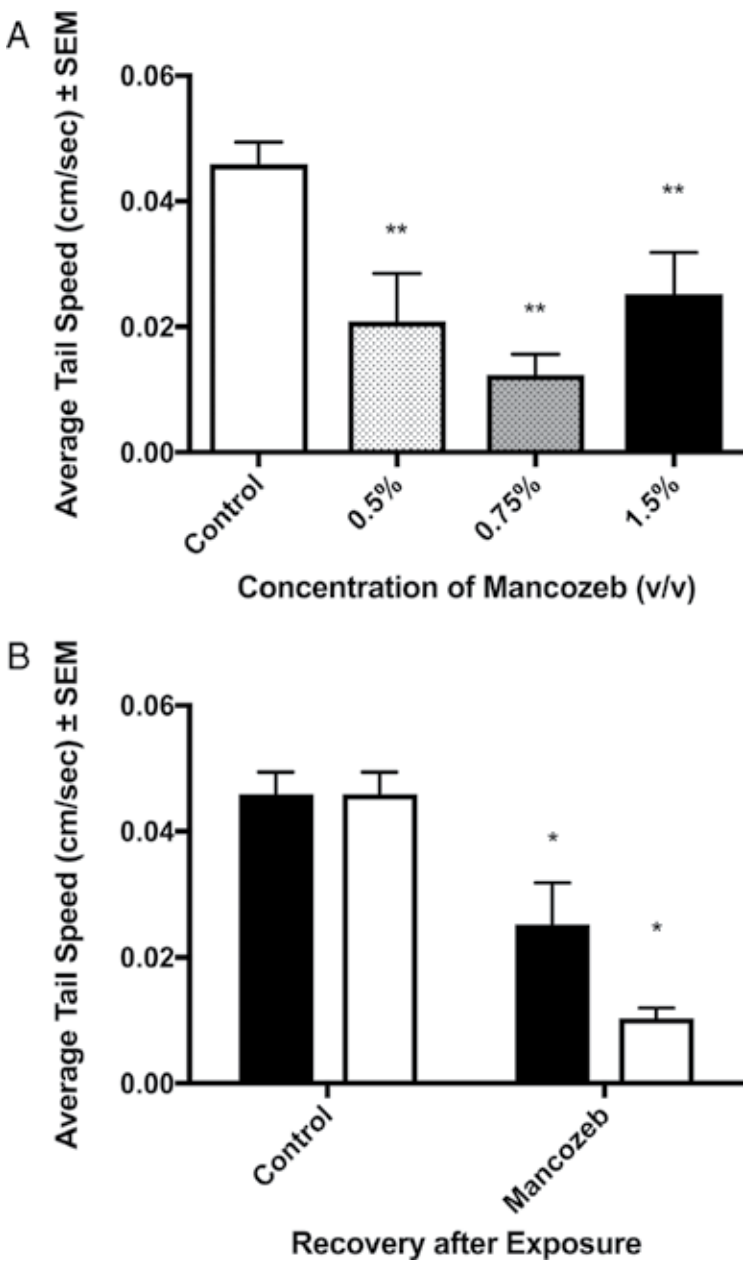

Figure 1. Long-lasting tail paralysis after exposure to Mancozeb. Tail speed after swimming in droplet of M9 buffer (as described more fully in Section 4; *n*= 12-21….replicates.) individual nematodes per treatment group across at least three separate replicates. (A) Immediately following Mancozeb exposure; (B) 24 h after Mancozeb exposure. Black bars indicate nematodes exposed to 1.5% Mancozeb for 24 h and assayed immediately thereafter. White bars are nematodes moved to untreated plates with food for 24 h after the Mancozeb exposure. Data are presented as the mean ± SEM. $^{**}p < 0.01$ when compared with untreated controls using one-way ANOVA followed by Bonferroni post-hoc test for multiple comparisons.

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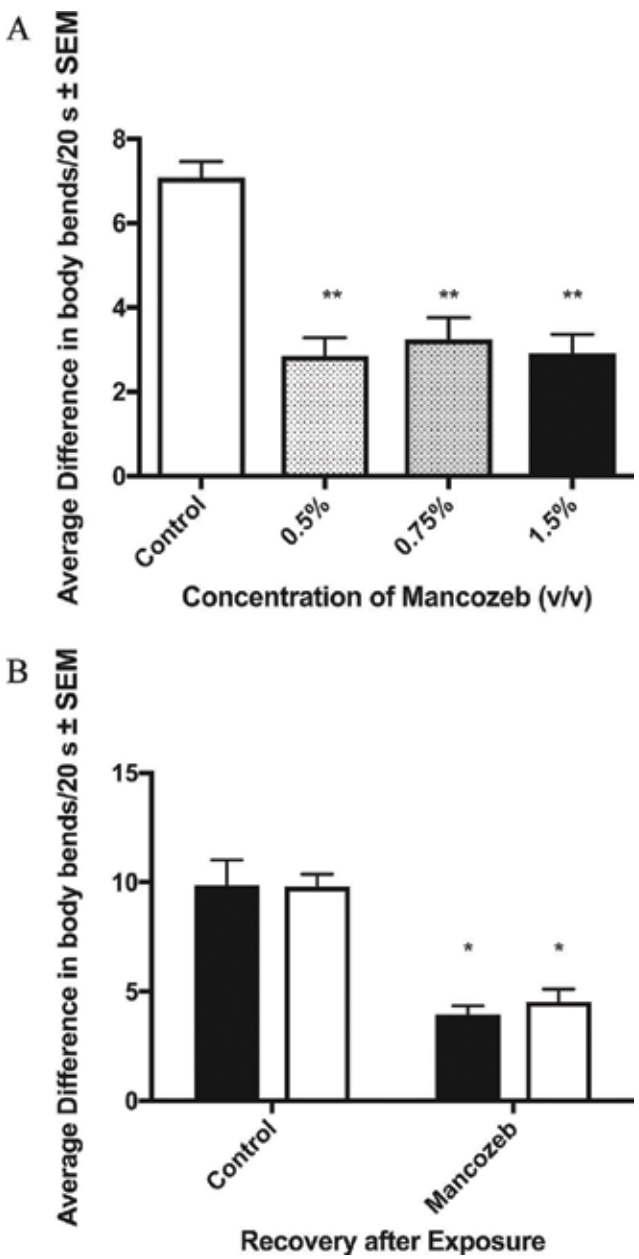

Figure 2. Basal slowing behavior impairments after 24 h exposure to 1.5% Mancozeb are not reversible after 24 h recovery. Day 4 nematodes at the L4/adult transition were exposed to 1.5% Mancozeb for 24 h and then basal slowing behavior was measured as the change in body bends upon entry to a bacterial lawn. Black bars indicate worms analyzed immediately following exposure to 1.5% Mancozeb. White bars show nematodes treated and then measured immediately following exposure (A) or moved to untreated plates with food for a 24 h recovery period prior to measurement (B). Data are averages of $n = 20$ independent worms per treatment across three separate replications. *$p < 0.01$ when compared with time-matched controls using a 2-way ANOVA followed by Bonferroni multiple comparisons tests.

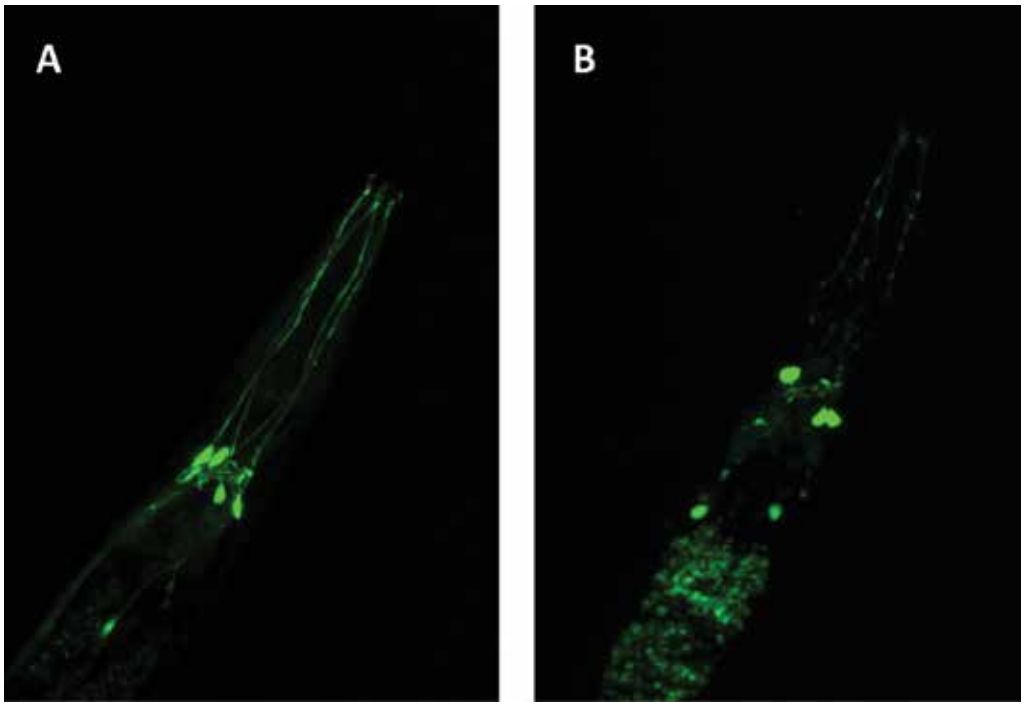

Figure 3. Mancozeb-induced degeneration of dopamine neurons. Late L4/young adult nematodes of the OH7547 strain, which express GFP in dopamine neurons were exposed to 1.5% Mancozeb as described in Section 4. Individual nematodes were picked to 2% agarose pads in a droplet of M9 buffer containing 2.5% NaN_3 to paralyze them and viewed using confocal microscopy. (A) Untreated nematode. The two pairs of CEP neurons and one of the ADE neurons are visible, along with their long processes. (B) Nematode exposed to 1.5% Mancozeb. 60× oil immersion lens.

6. Conclusions

Dithiocarbamate fungicides are widely used globally in large part because they are thought to be safe for wildlife and humans [7]. However, there is growing evidence that these compounds, and their breakdown products, harm wildlife and may even lead to Parkinson's like symptoms in humans [62, 63]. Further, a number of reports indicate that these compounds in combination with other widely used pesticides like paraquat or several organophosphate pesticides [64] are associated with cellular and organismal toxicity.

Mancozeb and maneb, the two most commonly used dithiocarbamate fungicides, have adverse effects on a number of non-target organisms, including protozoans [65], soil arthropods [66], birds [67], rodents [68] and fish [69]. The toxic effects include neurotoxicity [7], immunotoxicity, reproductive defects and endocrine disruption [67] for both high doses and low doses. Even though these compounds have a relatively short half-life, the metabolites and the metal ions persist in the environment. In addition, in part because of the short half-life, farmers and gardeners tend to apply the pesticides multiple times during a growing season. Further, the behavioral damage we documented in *C. elegans* is permanent and occurs well

within the active life of the pesticides. As a result, exposure to the compounds and their toxic byproducts is far greater and far more long-lasting, particularly during growing seasons, which correspond to breeding, growing and active periods of many organisms.

These studies have underscored the utility of examining the effects of commonly used fungicides like the dithiocarbamates and metal-containing compounds (particularly zinc and manganese) on *C. elegans* behavior, development and neural function. More recently, researchers evaluated the effects of triazole fungicides, particularly epoxiconazole, on nematode growth and reproduction [18] and determined that spermatogenesis is inhibited by epoxiconazole. These findings agree with reports of endocrine-disrupting effects of this family of fungicides in rats and birds [17, 70, 71]. The advantages of short life span, comprehensive genetics and ease of study make these nematodes an ideal system for continued study of the effects of fungicides, necessary agents for agriculture and medicine, on organismal development, health and behavior.

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References

[1] Moore D, Robson GD, Trinci APJ. 21st Century Guidebook to Fungi. Cambridge, UK: Cambridge University Press; 2011 ISBN: 9780521186957

[2] Gilliam M. Factors affecting development of chalkhood disease in colonies of honey bees, *Apis mellifera*, fed pollen contaminated with *Ascosphaera apis*. Journal of Invertebrate Pathology. 1988;**52**:314-325

[3] Kuivila KM, Hladik ML, Ingersoll CG, Kemble NE, Moran PW, Calhoun DL, Nowell LH, Gilliom RJ. Occurrence and potential sources of pyrethroid insecticides in stream sediments from seven U.S. metropolitan areas. Environmental Science and Technology. 2012;**46**:4297-4303

[4] Grun F, Blumberg B. Perturbed nuclear receptor signalling by environmental obesogens as emerging factors in the obesity crisis. Reviews in Endocrine & Metabolic Disorders. 2007;**8**:161-171

[5] Freire C, Koifman S. Pesticide exposure and Parkinson's disease: Epidemiological evidence of association. Neurotoxicology. 2012;**33**:947-971

[6] Rachid Rouabhi (2010). Introduction and Toxicology of Fungicides, Fungicides, Odile Carisse (Ed.), pp. 363-382. ISBN: 978-953-307-266-1, Croatia, InTech. Available from: http://www.intechopen.com/books/fungicides/introduction-and- toxicology-of-fungicides

[7] Domico LM, Zeevalk GD, Bernard LP, Cooper KR. Acute neurotoxic effects of mancozeb and maneb in mesencephalic neuronal cultures are associated with mitochondrial dysfunction. Neurotoxicology. 2006;**27**:816-825

[8] Bjorling-Poulsen M, Andersen H, Grandjean P. Potential developmental neurotoxicity of pesticides used in Europe. Environmental Health. 2008;**7**:50-72. https://doi.org/10.1186/1476-069X-7-50

[9] Bisenius ES, Veeramachaneni DNR, Sammonds GE, Tobet S. Sex differences and the development of the rabbit brain: Effects of vinclozolin. Biology of Reproduction. 2006;**75**:469-476

[10] Cabral SMJCS, Cabral JPS. The primary mode-of-action of vinclozolin: Are oxygen free radicals directly involved? Pesticide Biochemistry and Physiology. 2000;**66**:145-152

[11] Schmuck G, Mihail F. Effects of carbamates fenoxycarb, propamocarb and propoxur on energy supply, glucose utilization and SH-groups in neurons. Archives of Toxicology. 2004;**78**:330-337

[12] Morton V, Staub T. (2008) A short history of fungicides. The American Phytopathological Society. APS Features ISSN: 2153-0297 DOI: 10.1094/APSnetFeature-2008-0308

[13] Faro LR. Neurotoxic effects of triazole fungicides on nigrostriatal dopaminergic neurotransmission. In: Carisse O. editor. Fungicides. INTECH; 2010. ISBN: 978-953-307-266-1. Available from: http://www.intechopen.com/books/fungicides/neurotoxic-efects-of-triazole-fungicides-on-nigrostriataldopaminergic-neurotransmission

[14] Zaro JA, Brüschweiller BJ, Schlatter JR. Azole fungicides affect mammalian steroidogenesis by inhibiting sterol 14α-demethylase and aromatase. Environmental Health Perspectives. 2003;**111**:255-261

[15] Crofton KM, Boncek VM, Macphail RC. Evidence for monoaminergic involvement in triadimefom-induced hyperactivity. Psychopharmacology. 1989;**97**:326-330

[16] Rico A, Sabater C, Castillo M-A. Lethal and sub-lethal effects of five pesticides used in rice farming on the earthworm *Eisenia fetida*. Ecotoxicology and Environmental Safety. 2016;**127**:222-229

[17] Taxvig C, Hass U, Axelstad M, Dalgaard M, Boberg J, Andeason HR, Vinggaard AM. Endocrine-disrupting activities in vivo of the fungicides tebuconazole and epoxiconazole. Toxicological Sciences. 2007;**100**:464-473

[18] Li Y, Zhang M, Li S, Lv R, Chen P, Liu R, Liang G, Yin L. The use of the nematode *Caenorhabditis elegans* to evaluate the adverse effects of epoxiconazole exposure on spermatogenesis. International Journal of Environmental Research and Public Health. 2016;**13**:993-1005

[19] Wightwick A, Walters R, Allinson G, Reichman S, Menzies N. Environmental risks of fungicides used in horticultural production systems. In: Carisse O. editor. Fungicides. InTech; 2010. pp. 273-304. Available from: http://www.intechopen. com/books/fungicides/environmental-risks-of-fungicides-used-in-horticultural-production-systems

[20] Regueiro J, Olguin N, Simal-Gandara J, Sunol C. Toxicity evaluation of new agricultural fungicides in primary cultured cortical neurons. Environmental Research. 2015;**140**:37-44

[21] WHO. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues Geneva, Switzerland,15-24 September 2003. Rome: World Health Organization food and agriculture organization of the United Nations; 2004

[22] Von Stackelberg K. (2012) Potential health effects of azoxystrobin in the environment: A systematic review. pp. 2-22. http://www.ehrf.info/wp-content/uploads/2012/03/Azoxy-Systematic- Review.pdf

[23] Fishel FM. The EPA Conventional Reduced Risk Pesticide Program. Institute of Food and Agricultural Sciences Gainesville: University of Florida; 2010. pp. 1224-1228. http://edis.ifas.ufl.edu/pi224

[24] Parrón T, Requena M, Hernandez AF, Alarcon R. Association between environmental exposure to pesticides and neurodegenerative disease. Toxicology and Applied Pharmacology. 2011;**256**:379-385

[25] Raley-Susman KM. Like a canary in the coal mine: Behavioral change as an early warning sign of neurotoxicological damage. In: Larramendy ML, Soloneski S, editors. Pesticides Toxic Aspects. Croatia: InTech; 2014. p. 135-162

[26] Soleo L, Defazio G, Scarselli R, Zefferino R, Livrea P, Foa V. Toxicity of fungicides containing ethylene-bis-dithiocarbamate in serumless mesencephalic-striatal primary coculture. Archives of Toxicology. 1996;**70**:678-682

[27] Settivari R, LeVora J, Nass R. The divalent metal transporter homologues SMF-1/2 mediate dopamine neuron sensitivity in *Caenorhabditis elegans* models of manganism and Parkinson disease. The Journal of Biological Chemistry. 2009;**284**:35758-35768

[28] Li P, Zhu J, Kong Q, Jiang B, Wan X, Yue J, Li M, Jiang H, Li J, Gao J. The ethylene-bis-dithiocarbanate fungicide mancozeb activates voltage-gated KCNQ2 potassium channel. Toxicology Letters. 2013;**219**:211-217

[29] Domico LM, Cooper KR, Bernard LP, Zeevalk GD. Reactive oxygen species generation by the ethylene-bis-dithiocarbamate (EBDC) fungicide mancozeb and its contribution to neuronal toxicity in mesencephalic cells. Neurotoxicology. 2007;**28**:1079-1091

[30] Bailey DC, Todt CE, Orfield SE, Denney RD, Snapp IB, Negga R, Montgomery KM, Bailey AC, Pressley AS, Traynor WL, Fitsanakis VA. *Caenorhabditis elegans* chronically exposed to a Mn/Zn ethylene-bis-dithiocarbamate fungicide show mitochondrial Complex I inhibition and increased reactive oxygen species. Neurotoxicology. 2016;**56**:170-179

[31] Avila D, Helmcke K, Aschner M. *Caenorhabditis elegans* model as a reliable tool in neurotoxicology. Human and Experimental Toxicology. 2012;**31**:236-243

[32] Horiuchi J, Prithiviraj B, Bais HP, Kimball BA, Vivanco JM. Soil nematodes mediate positive interactions between legume plants and rhizobium bacteria. Planta. 2005;**222**:848-857

[33] Brody HA, Chou E, Gray JM, Pokrywka NJ, Raley-Susman KM. Mancozeb-induced behavioral deficits precede structural neural degeneration. Neurotoxicology. 2013; **34**:74-81

[34] Neher DA. Role of nematodes in soil health and their use as indicators. Journal of Nematology. 2001;**33**:161-168

[35] Israeli R, Sculsky M, Tiberin P. Acute central nervous system changes due to intoxication by Manzidan (a combined dithiocarbamate of Maneb and Zineb). Archives of Toxicology. 1983;**6**:238-243

[36] Ferraz HB, Bertolucci PHF, Pereira JS, Lima JGC, Andrade LAF. Chronic exposure to the fungicide Maneb may produce symptoms and signs of CNS manganese intoxication. Neurology. 1988;**38**:550-553

[37] Meco G, Bonifati V, Vanacore N, Fabrizio E. Parkinsonism after chronic exposure to the fungicide Maneb (Manganese ethylene-bis-dithiocarbamate). Scandinavian Journal of Work, Environment & Health. 1994;**20**:301-305

[38] Sulston J, Dew M, Brenner S. Dopaminergic neurons in the nematode *Caenorhabditis elegans*. The Journal of Comparative Neurology. 1975;**163**:215-226

[39] Sawin ER, Ranganathan R, Horvitz HR. *C. elegans* locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway. Neuron. 2000;**26**:619-631

[40] Vidal-Gadea A, Topper S, Young L, Crisp A, Dressin L, Elbel E. *Caenorhabditis elegans* selects distinct crawling and swimming gaits via dopamine and serotonin. Proceedings of the National Academy of Sciences of the United States of America. 2011;**108**:17504-17509

[41] Lints R, Emmons SW. Patterning of dopaminergic neurotransmitter identity among *Caenorhabditis elegans* ray sensory neurons by a TGF family signaling pathway and a Hox gene. Development. 1999;**126**:5819-5831

[42] Calhoun AJ, Tong A, Pokala N, Fitzpatrick JAJ, Sharpee TO, Chalasani SH. Neural mechanisms for evaluating environmental variability in *Caenorhabditis elegans*. Neuron. 2015;**86**:428-441

[43] Ailion M, Thomas JH. Dauer formation induced by high temperatures in *Caenorhabditis elegans*. Genetics. 2000;**156**:1047-1067

[44] Hills T, Brockie PJ, Maricq AV. Dopamine and glutamate control area-restricted search behavior in *Caenorhabditis elegans*. The Journal of Neuroscience. 2004;**24**:1217-1225

[45] Sanyal S. Dopamine modulates the plasticity of mechanosensory responses in *Caenorhabditis elegans*. The EMBO Journal. 2004;**23**:473-482

[46] Kindt KS. Dopamine mediates context-dependent modulation of sensory plasticity in *C. elegans*. Neuron. 2007;**55**:662-676

[47] Voglis G, Tavernarakis N. A synaptic DEG/ENaC ion channel mediates learning in *C. elegans* by facilitating dopamine signalling. The EMBO Journal. 2008;**27**:3288-3299

[48] Kimura KD, Fujita K, Katsura I. Enhancement of odor avoidance regulated by dopamine signaling in *Caenorhabditis elegans*. The Journal of Neuroscience. 2010;**30**:16365-16375

[49] Tsang MM, Trombetta LD. The protective role of chelators and antioxidants on mancozeb-induced toxicity in rat hippocampal astrocytes. Toxicology and Industrial Health. 2007;**7**:59-470

[50] Gullino M-L, Tinivella R, Garibaldi A, Kemmitt GM, Bacci L, Sheppard B. Dithiocarbamate-containing pesticides lead to degeneration of ∂-aminobutyric acid and dopamine neurons in *Caenorhabditis elegans*. Neurotoxicity Research. 2010;**21**:281-290

[51] Shukla Y, Arora A. Transplacental carcinogenic potential of the carbamate fungicide mancozeb. Journal of Environmental Pathology, Toxicology, and Oncology. 2001;**20**:127-131

[52] Cuajungco MP, Lees GJ. Zinc metabolism in the brain: Relevance to human neurodegenerative disorders. Neurobiology of Disease. 1997;**4**:137-169

[53] Aschner M, Guilarte TR, Schneider JS, Zheng W. Manganese: Recent advances in understanding its transpot and neurotoxicity. Toxicology and Applied Pharmacology. 2007;**221**:131-147

[54] Benedetto A, Au C, Avila DS, Milatovic D, Aschner M. Extracellular dopamine potentiates Mn-induced oxidativee stress, lifespan reduction, and dopaminergic neurodegeneration in a BLI-3-dependent manner in *Caenorhabditis elegans*. PLOS Genetics. 2010;**6**:1-17

[55] Negga R, Rudd DA, Davis NS, Justice AN, Hatfield HE, Valente AL. Exposure to Mn/Zn ethylene-bis-dithiocarbamate and glyphosate pesticides leads to neurodegeneration in *Caenorhabditis elegans*. Neurotoxicology. 2011;**32**:331-341

[56] Easton A, Guven K, de Pomerai DI. Toxicity of the dithiocarbamate fungicide Mancozeb to the nontarget soil nematode *Caenorhabditis elegans*. Journal of Biochemical and Molecular Toxicology. 2001;**15**:15-25

[57] Ruan Q-L, Ju J-J, Li Y-H, Liu R, Pu Y-P, Yin L-H. Evaluation of pesticide toxicities with differing mechanisms using *Caenorhabditis elegans*. Journal of Toxicology and Environmental Health. 2009;**72**:746-751

[58] Mergler D. Neurotoxic effects of low level exposure to manganese in human populations. Environmental Research. 1999;**80**:99-102

[59] Stiernagle T. Maintenance of *C. elegans* (February 11, 2006). In: Wormbook, editor. The *C. elegans* Research Community. WormBook; 2006. DOI: 10.1895/wormbook.1.101.1 http://www.wormbook.org

[60] Hart AC. Behavior. In: WormBook, editor. The *C. elegans* Research Community. 2006. DOI: 10.1895/wormbook.1.87.1 http://www.wormbook.org

[61] Negga R, Stuart JA, Machen ML, Salva J, Sizek AJ, Richardson SJ. Exposure to glyphosate- and/or Mn/Zn-ethylene-bis-dithiocarbamate-containing pesticides leads to degeneration of g-aminobutyric acid and dopamine neurons in *Caenorhabditis elegans*. Neurotoxicity Research. 2012;**21**:281-290

[62] Debbarh I, Rambelomanana S, Penouil F, Castaigne F, Poisot D, Moore N. Human neurotoxicity of ethylene-bis-dithiocarbamates (EBDC). Revue Neurologique. 2002; **158**:1175-1180

[63] Moretto A, Colosio C. The role of pesticide exposure in the genesis of Parkinson's disease: Epidemiological studies and experimental data. Toxicology. 2013;**307**:24-34

[64] Cicchetti F, Lapointe N, Roberge-Tremblay A, Saint-Pierre M, Jimenez L, Ficke BW. Systemic exposure to paraquat and maneb models early Parkinson's disease in young adult rats. Neurobiology of Disease. 2005;**20**:360-371

[65] Bonnemain H, Dive D. Studies on synergistic toxic effects of dithiocarbamate pesticides with the ciliate protozoan *Colpidium campylum* (Stokes). Ecotoxicology and Environmental Safety. 1990;**19**:320-326

[66] Adamski Z, Bloszyk J, Piosik K, Tomczak K. Effects of diflubenzuron and mancozeb on soil microarthropods: A long-term study. Biology Letters. 2009;**46**:3-13

[67] Pandey SP, Mohanty B. The neonicotinoid pesticide imidacloprid and the dithiocarbamate fungicide Mancozeb disrupt the pituitary-thyroid axis of a wildlife bird. Chemosphere. 2015;**122**:227-234

[68] Bhaskar R, Mohanty B. Pesticides in mixture disrupt metabolic regulation: In silico and in vivo analysis of cumulative toxicity of Mancozeb and imidacloprid on body weight of mice. General and Comparative Endocrinology. 2014;**205**:226-234

[69] Van Boxtel A, Kamstra JH, Fluitsma DM, Legler J. Dithiocarbamates are teratogenic to developing zebrafish through inhibition of lysyl oxidase activity. Toxicology and Applied Pharmacology. 2010;**244**:156-161

[70] Hass U, Boberg J, Christiansen S, Jacobsen PR, Vinggaard AM, Taxvig C, Poulsen ME, Herrmann SS, Jensen BH, Petersen A. Adverse effects on sexual development in rat offspring after low does exposure to a mixture of endocrine disrupting pesticides. Reproductive Toxicology. 2012;**34**:261-274

[71] Stinchcombe S, Schneider S, Fegert I, Rey Moreno MC, Strauss VC, Groters S, Fabian E, Fussell KC, Pigott GH, van Ravenzwaay B. Effects of estrogen coadministration on epoxiconazole toxicity in rats. Birth Defects Research. Part B, Developmental and Reproductive Toxicology. 2013;**98**:247-259

Chapter 4

Neurotoxins and Autism

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Additional information is available at the end of the chapter

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Abstract

Recently, a great concern has risen about the increasing prevalence of autism as a neurodevelopmental disorder. Environmental factors as significant contributors to children's health through a wide range of routes are linked to remarkable increases in this disorder. It is well known and accepted that young children are more vulnerable to environmental toxins, compared to adults. Modern day lifestyles with more mercury and lead exposures, fast food, cell phones, and microwaves place children at higher risk of neurotoxicity. Moreover, a huge number of synthetic chemicals termed as high-production-volume (HPV) chemicals are found in many products such as medications, cosmetics, building materials, plastic, and car fuels. These HPVs highly contribute to brain damage in developing infants. Other environmental toxins include thalidomide, valproic acid, misoprostol, and many infectious agents among which are pathogenic bacteria or their metabolites are found to be neurotoxic and/or linked to incidences of autism. This chapter summarizes the most important routes of exposure to environmental neurotoxins and explains how these toxins are related to the remarkable increase in the prevalence of autism through different etiological mechanisms such as oxidative stress, neuroinflammation, impaired neurochemistry and glutamate excitotoxicity.

Keywords: neurotoxins, heavy metals, mercury, *Clostridium difficile* aesthetic drugs, valproic acid, insecticides, herbicides, cell phones

1. Introduction

Recently, a great concern has risen about the increasing prevalence of "autism as a neurodevelopmental disorder" characterized by impaired social interaction, communication, and

repetitive behavior. Environmental factors as significant contributors to children's health through a wide range of routes are greatly involved in the remarkable increase of autism spectrum disorder (ASD). It is well known and accepted that young children are more vulnerable to environmental toxins compared to adults because they breathe more air and consume more food relative to their body size in order to meet requirements of growth and development. Common hand-to-mouth behavior among infants and even young children of course increases their risk of exposures to environmental toxins.

In relation to the modern lifestyles—cell phones, microwaves, mercury and lead exposures, plastic, fast food—place children at higher risk of neurotoxicity that might lead to brain damage during early development. Moreover, environmental toxins such as misoprostol, valproic acid (VPA), and thalidomide have been reported as neurotoxins and linked to ASD. Pathogenic bacteria or their metabolites, cytomegalovirus, rubella, toxoplasmosis, and herpes simplex have also been characterized as neurotoxins which greatly contribute to autism.

This chapter summarizes the most important routes of exposure to environmental neurotoxins and explains how these toxins are related to the remarkable increase in the prevalence of autism. Moreover, the role of the stages of development and timing of exposures (prenatal, perinatal, and postnatal) will be discussed.

Based on the recorded biomarkers of autism, the role of selected environmental toxins in the induction of oxidative stress, neuroinflammation, impaired neurochemistry, and glutamate excitotoxicity as etiological mechanisms related to autism will be highlighted and illustrated.

1.1. Heavy metals as neurotoxins

A relationship between rises in environmental levels of Hg and the increase in both rates of autism and special education students has been reported [1]. In an attempt to find the relationship between elevated mercury levels and oxidative stress as etiological mechanisms in autistic individuals, Sajdel-Sulkowska et al. [2] found that mercury concentrations in the cerebellar areas of the brain were positively correlated with neurotrophin-3 (NT-3), as an oxidative stress marker. Khan et al. reported on NT-3 associated with much higher levels in autistic patients but without an association with Hg levels in blood, which was nonsignificantly different between autistic and control subjects [3]. This suggests that the same concentration of Hg may promote oxidative stress only in autistic patients but not in control subjects. In relation to Hg levels in hair as an indicator of neurotoxicity in autistic patients, the "poor excretor theory" asserts that autistic children are more prone to accumulate Hg because they are unable to readily excrete it when compared to age- and gender-matched controls [4]. However, other studies recorded that the higher the Hg levels in hair, the worse the autism symptoms [5]. A direct relationship between elevated blood levels of Hg and the degree of autism severity according to scales childhood autism rating scales (CARS), social responsiveness scales (SRS), and short sensory profile (SSP) was ascertained [6, 7].

In an attempt to better understand the role of heavy metal neurotoxicity, investigations of air pollution, Hg, lead (Pb), and arsenic (As) have been shown to stimulate oxidative stress and

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inflammation in humans, which may contribute to the pathogenesis of autism [8, 9]. Based on multiple studies, Pb, Hg, and As were recorded as neurotoxins related to autism [10–12]. Associations between autism prevalence and proximity to industrial facilities were ascertained by Dickerson et al. [13]. Most recently, a disruption of complex neuro-immune signaling as a mechanism necessary for neuronal migration and brain growth was accepted to be a possible mechanism to cause Hg-induced brain damage [14].

Gut microbiota, which is known to be remarkably modulated in autistic patients, can be easily related to the elevated level of Hg. Gut microbes can modulate Hg via either methylation of less toxic inorganic Hg, Hg^{+2}, or demethylation (i.e., detoxification) of methylmercury (MeHg) [15–18]. In studying bacterial diversity in relation to the MeHg level, Rothenberg et al. found that, among the studied bacterial species *Clostridiales*, *Subdoligranulum*, and *Akkermansia* spp., positive correlations for stool MeHg, hair total mercury (THg), and stool inorganic Hg were evident, while negative correlations for *Streptococcus* were determined using Spearman's and/or Pearson's [19]. These relative effects were related to approximately tenfold higher *Clostridium difficile* levels in the stool from autistic subjects, which may help to support the use of probiotics to ameliorate MeHg elevations which may lead to the development of autistic features [20]. This is summarized in **Figure 1**.

In relation to the antioxidant and protective effect of selenium (Se), a recent review was written by Bjorklund and Causey on the molecular interactions between Hg and Se which result in neurotoxicity. Selected studies revealed associations between autism and Hg and Se concentration changes in hair and/or nails of autistic patients [21]. These studies reported significant increases in the levels of Hg and concurrent decreases in the levels of Se in hair and nails and

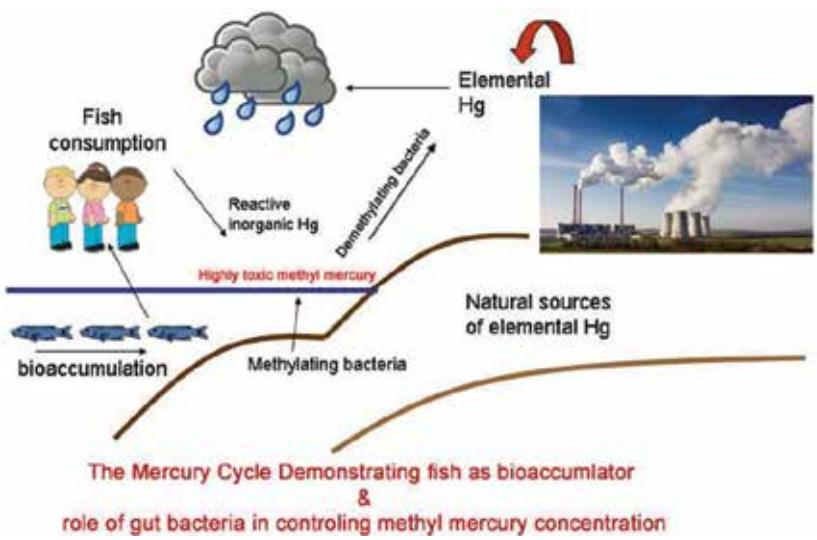

Figure 1. Role of fish in the bioaccumulation of mercury (Hg) and role of methylating and demethylating bacteria in the control of methyl mercury (MeHg) concentration as environmental neurotoxin related to autism.

the association of these changes with the severity of an autistic phenotype [5, 22]. Other studies showed significant elevations in Hg in hair [23] and urine [22] of autistic patients without significant decrease of Se concentration or Se/Hg ratio. Moreover, some studies reported a lower Zn/Cu ratio in blood from autistic subjects as compared to healthy control subjects [24, 25] yet strong causal relationships for Hg neurotoxicity.

1.2. Anesthetic drugs as neurotoxins in autism

An early study of the role of anesthesia in relation to neurotoxicity was described by Ikonomidou et al. [26]. They observed the effects of N-methyl-D-aspartate (NMDA) antagonist injections in rat pups, which led to acute postnatal neuronal apoptosis [26]. Also, they hypothesized that anesthetics such as ketamine blocked endogenous glutamate stimulation via NMDA receptors, leading to neuronal apoptosis. Apoptosis of neurons as an invasive marker of neurotoxicity was repeatedly demonstrated especially in animals that received multiple, high doses of ketamine during periods of developmental vulnerability [27, 28]. Upregulation of NMDA receptor expression levels in response to ketamine administration may modulate intracellular calcium homeostasis, possibly leading to apoptosis [29, 30]. Additionally, activation of gamma-aminobutyric acid (GABA) receptors through the inhalation of isoflurane-induced neurotoxicity in hippocampal culture cells was also associated with excessive neuronal influx of calcium [31]. Based on observation, it was suggested that anesthesia-induced neuronal toxicity may appear secondary to loss of calcium homeostasis within mitochondria as evident by mitochondrial dysfunction, accumulation of reactive oxygen species (ROS), and overexpression of caspases as pro-apoptotic markers (**Figure 2**) [32–34]. Although the actual mechanism for ROS accumulation was not identified, administration(s) of either an antioxidant or a

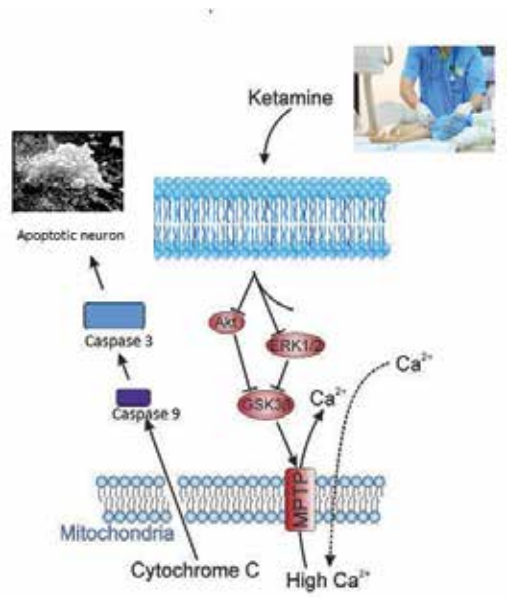

Figure 2. Neurotoxic effect of ketamine through loss of calcium homeostasis leading to neuronal death.

mitochondrial protectant prevented anesthesia-induced neuronal apoptosis and downstream cognitive impairment in developing rat brains [33, 35].

1.3. Antiepileptic drugs (AEDs) as neurotoxins in autism

The use of antiepileptic drugs (AEDs) by pregnant mothers was found to be involved in major congenital abnormalities seen in neurodevelopmental disorders among which is autism [36]. Animal studies demonstrated that exposure to AEDs may result in neurotoxicity; for example, VPA, phenytoin, and phenobarbital, cause impaired neurodevelopment after prenatal exposure [37]. Despite the therapeutic effects of VPA, it is also associated with neurotoxicity [38] as evidenced in in vitro models. VPA neurotoxicity is usually related to the increased of ROS production, as a critical contributor to brain damage and dysfunction [38–40]. Mitochondrial dysfunction has been proposed as one of the most common deleterious effects of VPA neurotoxicity [41]. In relation to autism, VPA is most commonly related to the etiopathology and development of most of phenotypic features of autism. Many cases, population database studies, prospective studies, and retrospective studies, ascertained increased incidences of ASD and cognitive deficits in VPA-exposed children with a reported risk of 6–8% [42–45].

Chaudhary and Parvez observed a significant decrease in acetylcholinesterase (AChE) activity [46]. The inhibition of AChE activity by VPA in the cerebellum and cerebral cortex results in the accumulation of ACh at cholinergic synapses, leading to ACh receptor stimulation, decreased cellular metabolism, induction of cell membrane alterations, and disturbances in neuronal activities [47]. VPA-inhibited Na^+/K^+-ATPase is an enzyme controlling the active transport of CNS sodium and potassium ions in a dose-dependent manner [48]. The marked inhibition of Na^+/K^+-ATPase activity may compromise neurotransmission, leading to partial membrane depolarization and excessive Ca^{2+} entry inside neurons which may in turn induce glutamate excitotoxicity. This can be ascertained through the recent work of Kim et al. in which agmatine was used to treat VPA-induced animal models of neurotoxicity, whereto, the amelioration of glutamate excitability improves sociability and decreases the repetitive behavior appeared as two important autistic features [49]. In a recent study by Videman et al., carbamazepine, oxcarbazepine, and VPA were associated with impaired early language abilities at the age of 7 months. In contrast, face perception or social attention may be less affected by the neurotoxic effects of the studied AEDs [50]. Previously, the association between prenatal exposure to AEDs and the increased risk of cognitive impairment and ASD was detected at ages of 2 to 6 years.

1.4. Clostridium neurotoxins and autism

It is well accepted that healthy gut microbiota provides an effective barrier against colonization by opportunistic bacteria [51, 52]. This protective microbiota is severely disrupted with abuse of broad-spectrum antibiotics frequently administered during early childhood [53]. *C. difficile* produces two exotoxins: toxin A and toxin B. Acting together, these toxins damage intestinal mucosa and cells and result in watery diarrhea, which is the primary clinical symptom of *C. difficile* infection [54]. In addition to *C. difficile*, *Clostridium tetani* is another opportunistic pathogen that can lay dormant in spore form for long periods of time. Both species

produce cytotoxins known to cause cellular damage. Additionally, both *C. tetani* and *C. difficile* produce phenolic metabolites [55]. Some toxigenic strains of *C. tetani* produce an extremely potent neurotoxin. Clostridia toxins can enter the circulation and accumulate at nerve terminals through binding to host-independent receptors [56, 57]. Following endocytosis of the neurotoxin-receptor complex, acidification of the presynaptic vesicle triggers a conformational change in the N-terminal translocation domain. Acidification is mediated by the vesicular ATPase proton pump, whose function is to ensure the reuptake of neurotransmitters into the synaptic vesicle. This might provide a plausible mechanism for the abnormal reuptake of glutamate in autistic patients. Several studies have assessed the fecal flora of autistic and control individuals, reporting an overgrowth of pathogenic bacterial species in autistic patients when compared to controls. Among these species is *Clostridium* species, including *C. difficile* [58–60]. In addition, Parracho et al. found a higher incidence of the *Clostridium histolyticum* group in the fecal flora of 58 ASD children compared to 10 healthy children [61].

Clayton hypothesized that impaired gut microbiota can be linked to autism through the abnormal gut bacterial metabolism of phenylalanine and tyrosine resulting in the production of P-cresol, with *C. difficile* as one of the most notable p-cresol producers [62]. Overgrowth of *C. difficile* can be linked to the etiology of autism through the inhibitory effect of p-cresol on dopamine β-hydroxylase as a rate-limiting enzyme of dopamine metabolism [63]. Moreover, *C. difficile-induced production of p-cresol can inhibit sulfonation as a detoxification mechanism of special importance when considering neonatal inactive* glucuronidation as an alternative detoxification reaction for xenobiotic excretion [64, 65]. Based on this information, p-cresol may be linked to autism through impaired gut microbiota and the overgrowth of *C. difficile* [66]. **Figure 3** summarizes the role of *C. difficile* overgrowth in the etiology of autism. Hsiao et al. reported that bacterial toxin-induced metabolic changes can trigger autistic behavior. Maternal immune-activated (MIA) females produce offspring

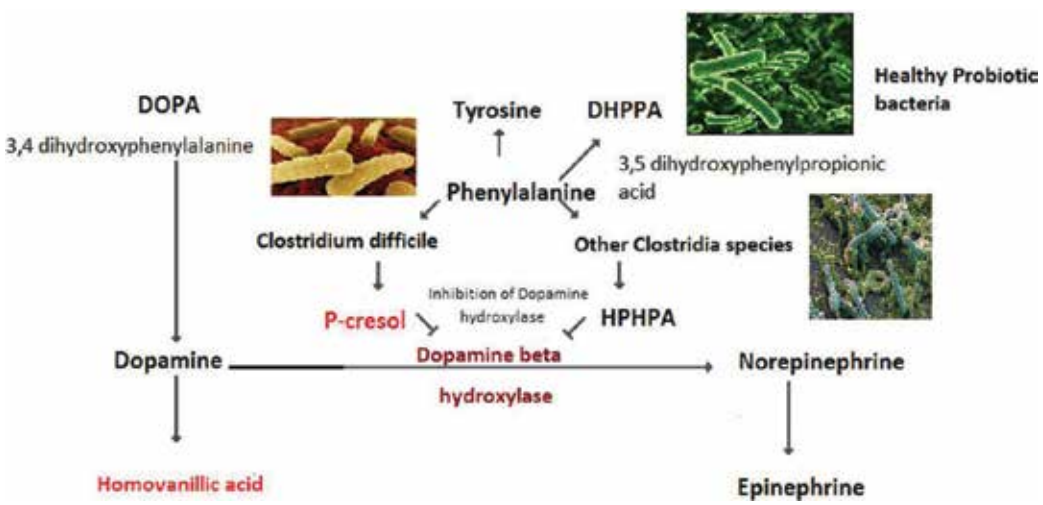

Figure 3. Role of *C.difficile* overgrowth in the etiology of autism through the inhibition of dopamine-beta hydroxylase by p-cresol as bacterial metabolite of tyrosine and phenylalanine.

with impaired communicative and social behavior representative as autistic features [67]. Moreover, MIA offspring displayed altered gut microbiota and leaky gut. Treatment of mice with *Bacteroides fragilis* was effective in restoring normal gut permeability and reduces anxiety-like behavioral deficits in this autism model [67]. Most recently, Yang and Chiu reported that through the soluble NSF attachment protein receptor (SNARE) complex, clostridial neurotoxins block neurotransmission to or from neurons. In addition, the gut microbiota produces molecules that act on enteric neurons to reduce gastrointestinal motility and metabolites that stimulate the "gut-brain axis" to alter neural circuits and brain function and behavior [68]. Aljarallah reported that water extract of myrrh plant demonstrates high antimicrobial effect against *C. difficile* strains, which could support its use as a natural product for the amelioration of *C. difficile* in autistic patients [69].

1.5. Pesticides as neurotoxins in autism

By using retrospective epidemiological studies, environmental factors such as pesticides have been linked to autism. Experimental research in mouse cortical neuron-enriched cultures exposed to hundreds of chemicals commonly found in the environment and on food showed how such chemicals greatly affect brain development. Pearson et al. have been found that rotenone, a pesticide associated with Parkinson's disease risk, and certain fungicides, including pyraclostrobin, trifloxystrobin, famoxadone, and fenamidone, produce transcriptional changes in vitro that are similar to those seen in brain samples from humans with autism [70]. These chemicals stimulate ROS production and disrupt microtubules in neurons, effects that can be reduced by pretreating with a microtubule stabilizer and an antioxidant such as sulforaphane. In this study, 283 autistic children showed neuronal tube defects with potential relationships between maternal residential proximity and agricultural use of neurotoxic pesticides [71].

Organophosphates (OPs) are the most generally utilized pesticides in agriculture, as well as bug sprays in residential, commercial, and industrial settings. Fetus may be exposed to OPs via the placenta or infants through breast milk, food, and inhalation. These small children appear particularly vulnerable to OPs and oxidative stress compared to adults, because of their lower activity levels of the enzyme paraoxonase, involved in OP inactivation and lipid peroxide degradation [72]. This enzyme was found to be significantly lower in autistic patients compared to healthy controls [73]. Prenatal exposure to OPs has been connected to neurodevelopmental disorder, which appears to be maintained during childhood, including deficits in cognitive abilities, working memory, and perceptual reasoning [74–76]. Attention deficits, receptive language, social cognition problems, reward, and behavioral dysfunction have been correlated with lower intelligence quotient (IQ) scores in humans prenatally exposed to chlorpyrifos [76]. Prenatal OP exposure has also been linked to increase autism risk [77]. Acetylcholinesterase (AChE) has been shown to be inhibited with OPs, determining excessive cholinergic transmission; however, OP's main neurotoxic actions are seemingly exerted by their axon metabolites [78].

There is a growing body of evidence that links the exposure to organochlorines (OCs) and autism. Despite their neurotoxic liabilities, OCs are frequently used in agriculture. The

association between autism and pesticide exposure during the third trimester has been observed from mothers living near agricultural areas where pesticides were used [79]. Shelton et al. found that increased exposure to insect repellent can lead to autism [80]. Eskenazi et al. and Rauh et al. reported that autistic children had higher OP metabolites during early to mid-pregnancy [77, 81]. Case-control studies reported that exposure to imidacloprid, insecticide, through the consistent use of flea/tick pet treatment throughout pregnancy period was associated with ASD [79, 82]. Rauh et al. reported that children exposed to OP insecticides showed psychomotor and mental development delays, attention, hyperactivity disorders, and pervasive developmental issues by 3 years of age [81].

Robert and English (2012) described ASD and applications of OCs in proximity to maternal residence before, during, and after pregnancy. Bayesian model as a flexible step function was formulated to measure the time that is needed by pesticide to affect fetus or children for mother who lived near agricultural area [83]. The association between autism and OCs was high, and the time of this association was extending from approximately 4 months prior to fertilization to 8 months into pregnancy. Roberts et al. have suggested that the risk of ASD is increased by 6.1-fold in children with maternal exposure to OCPs during the first trimester of pregnancy, a key period of gestation and neurodevelopmental processes in neonates [84]. Here, 465 children were enrolled in retrospective study to assess pesticide type, exposure time, and residential distance from pesticide application. This study reported an association between prenatal exposure to dicofol and endosulfan pesticide during the 8 weeks immediately following the time of cranial neural tube, and increased risk of autism in children of mothers who lived within fields that had the highest quartile of estimated pesticide exposure compared with children whose mothers lived more than far from exposure, and therefore had the lowest exposure levels [84].

Chlorinated biphenyl (CB) is used as dispersant in pesticide [85]. Because is a CB are pollutant with potentially persistent immunological and neurological effects [86, 87]. It has also been reported that CB can increase the production of ROS and cytotoxicity [88]. The neurological and immunological abnormalities as well as oxidative stress due to CB exposure have also been observed in autistic children [89–91]. **Figure 4** presents structures for selected insecticides linked to neurotoxicity and autism.

1.6. Endocrine-disrupting chemicals

Endocrine-disrupting chemicals (EDCs) such as polychlorinated biphenyl (PCB), polybrominated diphenyl ethers, bisphenol A (BPA), dioxins, and phthalate have strong associations with neurological disorders [92]. These EDCs are able to interfere with hormone functions because they may alter hormone-dependent processes and/or disrupt endocrine gland function. Certain EDCs are able to alter synaptic function and neural networks [93]. The EDCs are also termed neural-disrupting chemicals since they may increase the prevalence of neurodevelopmental disorders including autism. Prospective epidemiological studies are warranted to better understand EDC-related effects in humans [94]. Braun et al. found that midpregnancy BPA concentrations were associated with an increase in impaired neurodevelopment in early childhood [95]. In a study with 137 children, mothers with high phthalate metabolites in urine during the third trimester gave birth to infants that were more susceptible to

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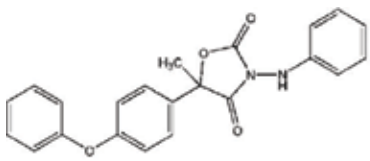
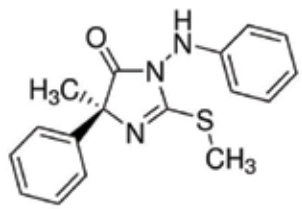


Famoxadone



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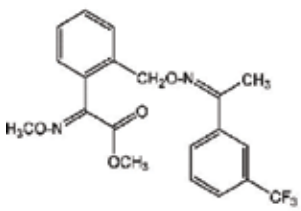
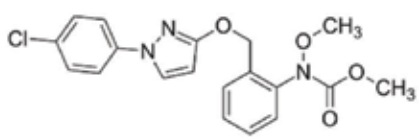
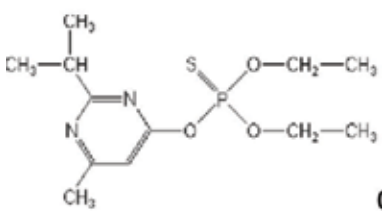


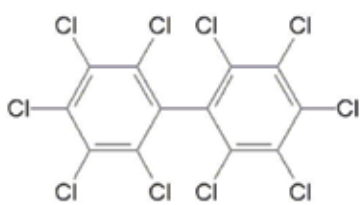
Trifloxystrobin



Pyraclostrobin



Organophosphorus



Chlorinated biphenyl

Figure 4. Structures of selected insecticides recorded as neurotoxin related to autism.

autism [96]. Larsson et al. have found a correlation between EDC and autism [97]. High serum phthalate concentrations have been recorded in autistic children [98]. Experimental animals show changes in development, synaptic organization, neurotransmitter synthesis and release, and brain structural organization due to exposure to EDC [99]. Cock et al. described links between brominated flame retardants, perfluorinated compounds, and ASD, yet additional weights of evidence are needed [100].

Several EDCs are known to disturb sex steroid and affect thyroid hormone (TH) levels which in turn are known to affect synaptogenesis, neuronal differentiation, migration, and myelination [101]. They play critical roles in brain development and potentially also for development of the connectome. TH receptor mutation in mice showed reduced density of GABAergic in the hippocampus, which was accompanied by more depressive and anxious behavior [102]. It is demonstrated that BPA inhibits the GABAAR-mediated response and that BPA affects development of GABAergic and dopaminergic systems [103]. Some studies indicate EDCs as a cause for neurodevelopmental disorders through GABAergic system changes [104, 105].

1.7. Radio frequency energy (RFE) of cell phone as neurotoxin in autism

During pregnancy, the possibility of fetal damage is increased as mothers are exposed to RFE [106]. Notably, the fetus may not be fully protected by amniotic fluid. It is well known that the pelvic structure permits deep penetration of the RFE to be absorbed within the developing fetus. Based on this, many investigations proposed that the dramatic increase in the incidence of autism since 1980 can be related to the neurotoxicity of cell phone radiation [107–109].

Based on our understanding on the etiological mechanisms in autism, such as oxidative stress, neuroinflammation, and glutamate excitotoxicity, the remarkable increase in the prevalence of autism about tenfolds since 1980 can be related to this dramatic increase which reach 1:45 on 2015 [110]. It is well documented that exposure to radio frequency radiation (RFR) can be accompanied by oxidative stress in human and animal models of autism [111, 112]. Unfortunately, these effects of cellular phone use can occur even at low and legal intensity which are now common environmental risk factors for infants, young children, adults, pregnant women, and fetuses. Cell phone radiations enhance free radical formation through the Fenton reaction as catalytic process through which iron converts hydrogen peroxides, a product of oxidative respiration in the mitochondria, into hydroxyl free radical, which is very potent and can induce damage of macromolecules, such as membrane phospholipids, DNA, and protein. Radio frequency radiation at very low intensities can also impair mitochondrial metabolism and modulate glutathione, glutamate, and GABA, which are substances related to the pathophysiology of autism [90, 113–116].

Fragopoulou et al. reported that through proteomic analysis of brain regulatory proteins from mice following prolonged exposure to electromotive force (EMF) led to either downregulation or overexpression of 143 proteins [117]. These altered proteins include neural function-related proteins, alpha-synuclein, glia maturation factor beta, cytoskeletal proteins, heat shock proteins, apolipoprotein E, as well as proteins of brain metabolism such as aspartate

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aminotransferase and glutamate dehydrogenase. These authors pointed out that oxidative stress was consistent with some changes in proteomic markers. Alteration in blood and brain glutathione status and deficiencies of reduced glutathione are increasingly associated with autism. Fortunately, certain studies demonstrating that supplementation with antioxidants such as vitamins C and E reduced oxidative impacts on rat endometrium from due to vitamins E and C reduced adverse impacts on rat endometrium due to exposure to 900 MHz EMR [118]. Ilhan et al. proved that *Ginkgo biloba* has also prevented mobile phone-induced increases in lipid peroxides and nitric oxide levels in brain tissue as well as decreases in brain superoxide dismutase and glutathione peroxidase activities and increases in brain xanthine oxidase and adenosine deaminase activities, together with the relief of the histopathological cell injury [119]. **Figure 5** demonstrates the role of cell phone radiation in the etiology of autism through oxidative stress as a major etiological mechanism.

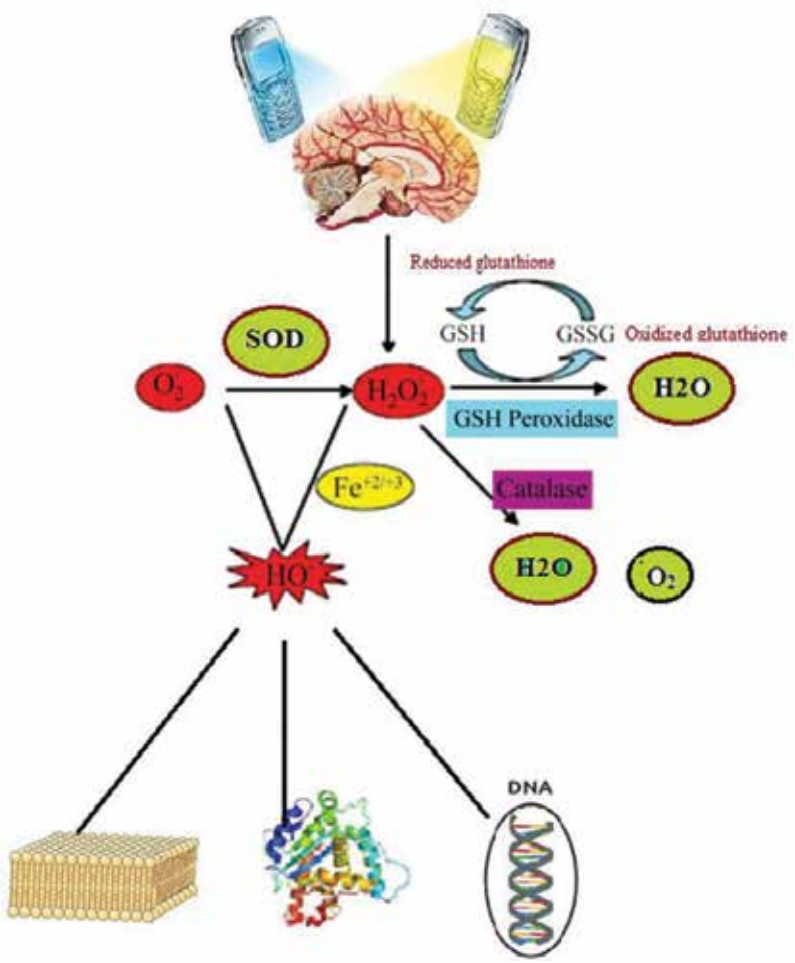

Figure 5. Role of *cell phone radiation* in the etiology of autism through oxidative stress as a major etiological mechanism.

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References

[1] Palmer RF, et al. Environmental mercury release, special education rates, and autism disorder: An ecological study of Texas. Health & Place. 2006;**12**(2):203-209

[2] Sajdel-Sulkowska EM. Brain development, environment and sex: What can we learn from studying graviperception, gravitransduction and the gravireaction of the developing CNS to altered gravity?. The Cerebellum. 2008;**7**(3):223-239

[3] Khan A, et al. Disrupted brain thyroid hormone homeostasis and altered thyroid hormone-dependent brain gene expression in autism spectrum disorders. Journal of Physiology and Pharmacology. 2014;**65**(2):257-272

[4] Holmes AS, Blaxill MF, Haley BE. Reduced levels of mercury in first baby haircuts of autistic children. International Journal of Toxicology. 2003;**22**(4):277-285

[5] Priya MDL, Geetha A. Level of trace elements (copper, zinc, magnesium and selenium) and toxic elements (lead and mercury) in the hair and nail of children with autism. Biological Trace Element Research. 2011;**142**(2):148-158

[6] Adams J, et al. The severity of autism is associated with toxic metal body burden and red blood cell glutathione levels. Journal of Toxicology. 2009;**2009**

[7] Alabdali A, Al-Ayadhi L, El-Ansary A. A key role for an impaired detoxification mechanism in the etiology and severity of autism spectrum disorders. Behavioral and Brain Functions. 2014;**10**(1):14

[8] Enstrom A, et al. Increased IgG4 levels in children with autism disorder. Brain, Behavior, and Immunity. 2009;**23**(3):389-395

[9] Li X, et al. Elevated immune response in the brain of autistic patients. Journal of Neuroimmunology. 2009;**207**(1):111-116

[10] Järup L. Hazards of heavy metal contamination. British Medical Bulletin. 2003;**68**(1): 167-182

[11] Sanders T, et al. Neurotoxic effects and biomarkers of lead exposure: A review. Reviews on Environmental Health. 2009;**24**(1):15-46

[12] Zheng W, Aschner M, Ghersi-Egea J-F. Brain barrier systems: A new frontier in metal neurotoxicological research. Toxicology and Applied Pharmacology. 2003;**192**(1):1-11

[13] Dickerson AS, et al. Autism spectrum disorder prevalence and proximity to industrial facilities releasing arsenic, lead or mercury. Science of the Total Environment. 2015;**536**:245-251

[14] Maqbool F, et al. Immunotoxicity of mercury: Pathological and toxicological effects. Journal of Environmental Science and Health, Part C. 2016 (just-accepted)

[15] Barkay T, Miller SM, Summers AO. Bacterial mercury resistance from atoms to ecosystems. FEMS Microbiology Reviews. 2003;**27**(2-3):355-384

[16] Gilmour CC, et al. Mercury methylation by novel microorganisms from new environments. Environmental Science & Technology. 2013;**47**(20):11810-11820

[17] Parks JM, et al. The genetic basis for bacterial mercury methylation. Science. 2013;**339** (6125):1332-1335

[18] Smith CC, et al. Dietary input of microbes and host genetic variation shape among-population differences in stickleback gut microbiota. The ISME Journal. 2015;**9**(11):2515-2526

[19] Rothenberg SE, et al. The role of gut microbiota in fetal methylmercury exposure: Insights from a pilot study. Toxicology Letters. 2016;**242**:60-67

[20] Navarro F, Liu Y, Rhoads JM. Can probiotics benefit children with autism spectrum disorders?. World Journal of Gastroenterology. 2016;**22**(46):10093

[21] Bjorklund DF, Causey KB. Children's Thinking: Cognitive Development and Individual Differences. SAGE Publications; 2017

[22] Blaurock-Busch E, et al. Toxic metals and essential elements in hair and severity of symptoms among children with autism. Maedica – A Journal of Clinical Medicine. 2012;**7**:38

[23] Al-Ayadhi L. Heavy metals and trace elements in hair samples of autistic children in central Saudi Arabia. Neurosciences (Riyadh, Saudi Arabia). 2005;**10**(3):213-218

[24] Faber S, et al. The plasma zinc/serum copper ratio as a biomarker in children with autism spectrum disorders. Biomarkers: Biochemical Indicators of Exposure, Response, and Susceptibility to Chemicals. 2009;**14**(3):171-180

[25] Cr*f*Éciun EC, et al. Evaluation of whole blood zinc and copper levels in children with autism spectrum disorder. Metabolic Brain Disease. 2016;**31**(4):887-890

[26] Ikonomidou C, et al. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science. 1999;**283**(5398):70-74

[27] Rudin M, et al. Single-dose ketamine administration induces apoptosis in neonatal mouse brain. Journal of Basic and Clinical Physiology and Pharmacology. 2005;**16**(4):231-244

[28] Slikker W, et al. Ketamine-induced neuronal cell death in the perinatal rhesus monkey. Toxicological Sciences. 2007;**98**(1):145-158

[29] Liu T, et al. Enhancement by interleukin-1β of AMPA and NMDA receptor-mediated currents in adult rat spinal superficial dorsal horn neurons. Molecular Pain. 2013;**9**(1):16

[30] Shi Q, et al. Gene expression profiling in the developing rat brain exposed to ketamine. Neuroscience. 2010;**166**(3):852-863

[31] Zhao C, Dreosti E, Lagnado L. Homeostatic synaptic plasticity through changes in presynaptic calcium influx. Journal of Neuroscience. 2011. **31**(20):7492-7496

[32] Yang J-L, et al. Mitochondrial DNA damage and repair in neurodegenerative disorders. DNA Repair. 2008;**7**(7):1110-1120

[33] Boscolo A, et al. The abolishment of anesthesia-induced cognitive impairment by timely protection of mitochondria in the developing rat brain: The importance of free oxygen radicals and mitochondrial integrity. Neurobiology of Disease. 2012;**45**(3):1031-1041

[34] Zhang Q, et al. Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature. 2010;**464**(7285):104-107

[35] Zanghi CN, Jevtovic-Todorovic V. A holistic approach to anesthesia-induced neurotoxicity and its implications for future mechanistic studies. Neurotoxicology and Teratology. 2016

[36] Tomson TR, et al. Antiepileptic drug exposure and major congenital malformations: The role of pregnancy registries. Epilepsy & Behavior. 2007;**11**(3):277-282

[37] Verrotti A, et al. Developmental neurotoxicity and anticonvulsant drugs: A possible link. Reproductive Toxicology. 2014;**48**:72-80

[38] Wang Z, et al. Valproic acid attenuates blood-brain barrier disruption in a rat model of transient focal cerebral ischemia: The roles of HDAC and MMP-9 inhibition. Journal of Cerebral Blood Flow & Metabolism. 2011;**31**(1):52-57

[39] Kiang TK, et al. Role of oxidative metabolism in the effect of valproic acid on markers of cell viability, necrosis, and oxidative stress in sandwich-cultured rat hepatocytes. Toxicological Sciences. 2010;**118**(2):501-509

[40] Zhang B, Wang X, Nazarali A. Ascorbic acid reverses valproic acid-induced inhibition of hoxa2 and maintains glutathione homeostasis in mouse embryos in culture. Cellular and Molecular Neurobiology. 2010;**30**(1):137-148

[41] Chang TK, Abbott FS. Oxidative stress as a mechanism of valproic acid-associated hepatotoxicity. Drug metabolism Reviews. 2006;**38**(4):627-639

[42] Bromley R, et al. Autism spectrum disorders following in utero exposure to antiepileptic drugs. Neurology. 2008;**71**(23):1923-1924

[43] Bromley RL, et al. The prevalence of neurodevelopmental disorders in children prenatally exposed to antiepileptic drugs. Journal of Neurology. Neurosurgery & Psychiatry. 2013;**84**(6):637-643

[44] Wood AG, et al. Prospective assessment of autism traits in children exposed to antiepileptic drugs during pregnancy. Epilepsia. 2015;**56**(7):1047-1055

[45] Christensen J, et al. Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. The Journal of the American Medical Association. 2013;**309**(16):1696-1703

[46] Chaudhary S, Parvez S. An in vitro approach to assess the neurotoxicity of valproic acid-induced oxidative stress in cerebellum and cerebral cortex of young rats. Neuroscience. 2012;**225**:258-268

[47] Roy R, Chaudhuri AN. Differential acetylcholinesterase activity in rat cerebrum, cerebellum and hypothalamus. Indian Journal of Experimental Biology. 2006;**44**(5):381-386

[48] Ribeiro CA, et al. Isovaleric acid reduces Na^+, K^+-ATPase activity in synaptic membranes from cerebral cortex of young rats. Cellular and Molecular Neurobiology. 2007;**27**(4):529-540

[49] Kim J-W, et al. Agmatine rescues autistic behaviors in the valproic acid-induced animal model of autism. Neuropharmacology. 2017;**113**:71-81

[50] Videman M, et al. Evidence for spared attention to faces in 7-month-old infants after prenatal exposure to antiepileptic drugs. Epilepsy & Behavior. 2016;**64**:62-68

[51] Holmes E, et al. Understanding the role of gut microbiome-host metabolic signal disruption in health and disease. Trends in Microbiology. 2011;**19**(7):349-359

[52] Kinross JM, Darzi AW, Nicholson JK. Gut microbiome-host interactions in health and disease. Genome Medicine. 2011;**3**(3):14

[53] Hentges DJ. The protective function of the indigenous intestinal flora. The Pediatric Infectious Disease Journal. 1986;**5**(1):17-20

[54] Tsutaoka B, et al. Antibiotic-associated pseudomembranous enteritis due to *Clostridium difficile*. Clinical Infectious Diseases. 1994;**18**(6):982-984

[55] Elsden SR, Hilton MG, Waller JM. The end products of the metabolism of aromatic amino acids by Clostridia. Archives of Microbiology. 1976;**107**(3):283-288

[56] Montecucco C. How do tetanus and botulinum toxins bind to neuronal membranes? Trends in Biochemical Sciences. 1986;**11**(8):314-317

[57] Simpson L, The life history of a botulinum toxin molecule. Toxicon. 2013;**68**:40-59

[58] Finegold SM, et al. Gastrointestinal microflora studies in late-onset autism. Clinical Infectious Diseases. 2002;**35**(Supplement 1):S6-S16

[59] Finegold SM, et al. Pyrosequencing study of fecal microflora of autistic and control children. Anaerobe. 2010;**16**(4):444-453

[60] Song Y, Liu C, Finegold SM. Real-time PCR quantitation of clostridia in feces of autistic children. Applied and Environmental Microbiology. 2004;**70**(11):6459-6465

[61] Parracho HM, et al. Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children. Journal of Medical Microbiology. 2005;**54**(10):987-991

[62] Clayton TA. Metabolic differences underlying two distinct rat urinary phenotypes, a suggested role for gut microbial metabolism of phenylalanine and a possible connection to autism. FEBS Letters. 2012;**586**(7):956-961

[63] Goodhart PJ, DeWolf WE Jr, Kruse LI. Mechanism-based inactivation of dopamine. beta.-hydroxylase by p-cresol and related alkylphenols. Biochemistry. 1987;**26**(9):2576-2583

[64] Alberti A, et al. Sulphation deficit in, "low-functioning" autistic children: A pilot study. Biological Psychiatry. 1999;**46**(3):420-424

[65] Coughtrie M. Sulfation through the looking glass-recent advances in sulfotransferase research for the curious. The Pharmacogenomics Journal. 2002;**2**(5):297-308

[66] Altieri L, et al. Urinary p-cresol is elevated in small children with severe autism spectrum disorder. Biomarkers: Biochemical Indicators of Exposure, Response, and Susceptibility to Chemicals. 2011;**16**(3):252-260

[67] Hsiao EY, et al. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell. 2013;**155**(7):1451-1463

[68] Yang NJ, Chiu IM. Bacterial signaling to the nervous system via toxins and metabolites. Journal of Molecular Biology. 2017;**429**

[69] Aljarallah KM. Inhibition of *Clostridium difficile* by natural herbal extracts. Journal of Taibah University Medical Sciences. 2016;**11**(5):427-431

[70] Pearson BL, et al. Identification of chemicals that mimic transcriptional changes associated with autism, brain aging and neurodegeneration. Nature Communications. 2016;**7**

[71] Gunier RB, et al. Prenatal residential proximity to agricultural pesticide use and IQ in 7-year-old children. Environ Health Perspect. 2016;**10**

[72] Furlong CE, et al. PON1 status of farmworker mothers and children as a predictor of organophosphate sensitivity. Pharmacogenetics and Genomics. 2006;**16**(3):183-190

[73] Paüca SP, et al. High levels of homocysteine and low serum paraoxonase 1 arylesterase activity in children with autism. Life Sciences. 2006;**78**(19):2244-2248

[74] Bouchard MF, et al. Prenatal exposure to organophosphate pesticides and IQ in 7-year-old children. Environmental Health Perspectives. 2011;**119**(8):1189

[75] Engel SM, et al. Prenatal exposure to organophosphates, paraoxonase 1, and cognitive development in childhood. Environmental Health Perspectives. 2011;**119**(8):1182

[76] Rauh V, et al. Seven-Year Neurodevelopmental Scores and Prenatal Exposure to Chlorpyrifos, A Common Agricultural Pesticide, in Everyday Environmental Toxins: Children's Exposure Risks. Apple Academic Press; 2015. pp. 201-219

[77] Eskenazi B, et al. Organophosphate pesticide exposure and neurodevelopment in young Mexican-American children. Environmental Health Perspectives. 2007;**115**:792-798

[78] Flaskos J. The developmental neurotoxicity of organophosphorus insecticides: A direct role for the oxon metabolites. Toxicology Letters. 2012;**209**(1):86-93

[79] Kalkbrenner AE, Schmidt RJ, Penlesky AC. Environmental chemical exposures and autism spectrum disorders: A review of the epidemiological evidence. Current Problems in Pediatric and Adolescent Health Care. 2014;**44**(10):277-318

[80] Shelton JF, et al. Neurodevelopmental disorders and prenatal residential proximity to agricultural pesticides: The CHARGE study. Environmental Health Perspectives (Online). 2014;**122**(10):1103

[81] Rauh VA, et al. Impact of prenatal chlorpyrifos exposure on neurodevelopment in the first 3 years of life among inner-city children. Pediatrics. 2006;**118**(6):e1845-e1859

[82] Keil AP, Daniels JL, Hertz-Picciotto I. Autism spectrum disorder, flea and tick medication, and adjustments for exposure misclassification: The CHARGE (CHildhood Autism Risks from Genetics and Environment) case-control study. Environmental Health. 2014;**13**(1):3

[83] Roberts EM, English PB. Bayesian modeling of time-dependent vulnerability to environmental hazards: An example using autism and pesticide data. Statistics in Medicine. 2013;**32**(13):2308-2319

[84] Roberts EM, et al. Maternal residence near agricultural pesticide applications and autism spectrum disorders among children in the California Central Valley. Environmental Health Perspectives. 2007;**115**:1482-1489

[85] Ritchie JM, et al. Organochlorines and risk of prostate cancer. Journal of Occupational and Environmental Medicine. 2003;**45**(7):692-702

[86] Crinnion WJ. Polychlorinated biphenyls: Persistent pollutants with immunological, neurological, and endocrinological consequences. Alternative Medicine Review. 2011;**16**(1)

[87] Kimura-Kuroda J, Nagata I, Kuroda Y. Disrupting effects of hydroxy-polychlorinated biphenyl (PCB) congeners on neuronal development of cerebellar Purkinje cells: A possible causal factor for developmental brain disorders? Chemosphere. 2007;**67**(9):S412-S420

[88] Zhu Y, et al. Polychlorinated-biphenyl-induced oxidative stress and cytotoxicity can be mitigated by antioxidants after exposure. Free Radical Biology and Medicine. 2009;**47**(12):1762-1771

[89] Chauhan A, Chauhan V. Oxidative stress in autism. Pathophysiology. 2006;**13**(3):171-181

[90] Chauhan VP, et al. Normalization of tumour blood vessels improves the delivery of nanomedicines in a size-dependent manner. Nature Nanotechnology. 2012;**7**(6):383-388

[91] Pardo-Villamizar CA, Zimmerman AW. Inflammation and neuroimmunity in the pathogenesis of autism: Neural and immune network interactions. In: Chauhan A, Chauhan V, Brown T, editors. Autism: Oxidative Stress, Inflammation, and Immune Abnormalities. 2009. pp. 225-244

[92] Herbstman JB, et al. Prenatal exposure to PBDEs and neurodevelopment. Environmental Health Perspectives. 2010;**118**(5):712

[93] Gilbert ME, et al. Developmental thyroid hormone disruption: Prevalence, environmental contaminants and neurodevelopmental consequences. Neurotoxicology. 2012;**33**(4): 842-852

[94] Kajta MÇ, Wójtowicz AK. Impact of endocrine-disrupting chemicals on neural development and the onset of neurological disorders. Pharmacological Reports. 2013;**65**(6): 1632-1639

[95] Braun JM, et al. Prenatal bisphenol A exposure and early childhood behavior. Environmental Health Perspectives. 2009;**117**:1945-1952

[96] Philippat C, et al. Phthalate concentrations in house dust in relation to autism spectrum disorder and developmental delay in the CHildhood Autism Risks from Genetics and the Environment (CHARGE) study. Environmental Health. 2015;**14**(1):56

[97] Larsson M, et al. Associations between indoor environmental factors and parental-reported autistic spectrum disorders in children 6-8 years of age. Neurotoxicology. 2009;**30**(5):822-831

[98] Kardas F, et al. Increased serum phthalates (MEHP, DEHP) and Bisphenol a concentrations in children with autism spectrum disorder: The role of endocrine disruptors in autism etiopathogenesis. Journal of Child Neurology. 2016;**31**(5):629-635

[99] Gore AC, Dickerson SM. Endocrine disruptors and the developing brain. In: Colloquium Series on The Developing Brain. Morgan & Claypool Life Sciences; 2012

[100] De Cock M, Maas YG, van de Bor M. Does perinatal exposure to endocrine disruptors induce autism spectrum and attention deficit hyperactivity disorders? Review. Acta Paediatrica. 2012;**101**(8):811-818

[101] Pilhatsch M, et al. Hypothyroidism and mood disorders: Integrating novel insights from brain imaging techniques. Thyroid Research. 2011;**4**(1):S3

[102] Alva-Sánchez C, Ortiz-Butrón RÃ, Pacheco-Rosado J. Kainic acid does not affect CA3 hippocampal region pyramidal cells in hypothyroid rats. Brain Research Bulletin. 2004;**63**(2):167-171

[103] Zhou R, et al. Abnormal synaptic plasticity in basolateral amygdala may account for hyperactivity and attention-deficit in male rat exposed perinatally to low-dose bisphenol-A. Neuropharmacology. 2011;**60**(5):789-798

[104] Antunes Fernandes EC, et al. Potentiation of the human GABAA receptor as a novel mode of action of lower-chlorinated non-dioxin-like PCBs. Environmental Science & Technology. 2009;**44**(8):2864-2869

[105] Roze E, et al. Prenatal exposure to organohalogens, including brominated flame retardants, influences motor, cognitive, and behavioral performance at school age. Environmental Health Perspectives. 2009;**117**:1953-1958

[106] Baste V, et al. Prospective study of pregnancy outcomes after parental cell phone exposure: The Norwegian Mother and Child Cohort Study. Epidemiology. 2015;**26**(4):613-621

[107] Byrd RH, Hribar ME, Nocedal J. An interior point algorithm for large-scale nonlinear programming. SIAM Journal on Optimization. 1999;**9**(4):877-900

[108] Bertrand J, et al. Prevalence of autism in a United States population: The Brick Township, New Jersey, investigation. Pediatrics. 2001;**108**(5):1155-1161

[109] Chakrabarti S, Fombonne E. Pervasive developmental disorders in preschool children. The Journal of the American Medical Association. 2001;**285**(24):3093-3099

[110] Young S, et al. A meta-analysis of the prevalence of attention deficit hyperactivity disorder in incarcerated populations. Psychological Medicine. 2015;**45**(2):247-258

[111] Phillips JL, Singh NP, Lai H. Electromagnetic fields and DNA damage. Pathophysiology. 2009;**16**(2):79-88

[112] Lai H. Evidence for Genotoxic Effects. The BioInitiative Working Group; 2007

[113] Brown HM, Oram-Cardy J, Johnson A. A meta-analysis of the reading comprehension skills of individuals on the autism spectrum. Journal of Autism and Developmental Disorders. 2013;**43**(4):932-955

[114] Choudhury PR, Lahiri S, Rajamma U. Glutamate mediated signaling in the pathophysiology of autism spectrum disorders. Pharmacology Biochemistry and Behavior. 2012;**100**(4):841-849

[115] Essa M, et al. Impaired antioxidant status and reduced energy metabolism in autistic children. Research in Autism Spectrum Disorders. 2013;**7**(5):557-565

[116] Coghlan S, et al. GABA system dysfunction in autism and related disorders: From synapse to symptoms. Neuroscience & Biobehavioral Reviews. 2012;**36**(9):2044-2055

[117] Fragopoulou AF, et al. Brain proteome response following whole body exposure of mice to mobile phone or wireless DECT base radiation. Electromagnetic Biology and Medicine. 2012;**31**(4):250-274

[118] Guney M, et al. 900 MHz radiofrequency-induced histopathologic changes and oxidative stress in rat endometrium: Protection by vitamins E and C. Toxicology and Industrial Health. 2007;**23**(7):411-420

[119] Ilhan A, et al. *Ginkgo biloba* prevents mobile phone-induced oxidative stress in rat brain. Clinica Chimica Acta. 2004;**340**(1):153-162

Chapter 5

Botulinum Neurotoxin: A Multifunctional Protein for the Development of New Therapeutics

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Additional information is available at the end of the chapter

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Abstract

Botulinum neurotoxin (BoNT) is a major therapeutic agent licensed in neurological indications such as dystonia and spasticity. In recent years, its use has steadily increased in other neurological areas and new therapeutic areas and also in the aesthetic setting. Paradoxically, BoNT is also the causative agent of the disease botulism and a potential bioterrorism toxin. The BoNT family of toxins comprised more than 40 individual members, classified into 7 serotypes and are produced by Gram-positive obligate anaerobic bacteria. BoNTs are enzymatic multi-modular proteins with a complex multistep mechanism of action. Their target site is at peripheral neurons, particularly the neuromuscular junction, at which they inhibit acetylcholine neurotransmission. Despite intense activity in the BoNT field, today there are still gaps in knowledge both in clinical practice and in basic research. The discovery of the structure-function of BoNT and its domains has allowed rational design of new features using molecular engineering. The diversity of BoNT molecules, both natural and engineered, is an invaluable pool from which to design future new therapeutics with unique pharmacological properties for current and novel indications.

Keywords: botulinum neurotoxin, therapeutic agent, botulism, recombinant protein, targeted secretion inhibitor

1. Introduction

There are currently four botulinum neurotoxin (BoNT) clinical products available in the Western hemisphere: abobotulinumtoxinA (Dysport®, Ipsen, Paris, France), incobotulinumtoxinA (Xeomin®, Merz Pharmaceuticals GmbH, Frankfurt, Germany), onabotulinumtoxinA (Botox®, Allergan, Irvine, CA, USA) and rimabotulinumtoxinB (Myobloc®, Solstice

Neurosciences, Louisville, KY, USA) [1]. Several other products are available for use in other countries, in particular in the Asian markets, and new formulations and products are under-development [2]. By 2022, it is expected that the market size for botulinum products will reach $6.6 billion, driven by the expansion of their therapeutic uses and also the appetite for non-invasive aesthetic applications [3].

Around 200 years ago (between 1817 and 1822), a German medical officer, Justinus Kerner, published a series of papers to provide the first accurate and complete description of the symptoms of food-borne botulism, which led to the discovery of BoNT as the causative agent and the prediction by Kerner of its potential clinical utility [4]. This fascinating class of proteins present a modular molecular architecture with distinct binding, translocation and enzymatic domains. The different structural and functional domains can be regarded as 'building blocks' and have facilitated a number of engineering approaches aimed, amongst other purposes, at extending the therapeutic applications of BoNTs to other cell types beyond their natural target of the neuromuscular junction [5].

The aim of this chapter is to (1) provide an overview of the current clinical uses and a historical perspective of botulinum neurotoxin discovery, the disease it causes and the threats and opportunities that it poses and (2) present the current understanding of the structure-function of the toxin and its application in the development of new therapeutics.

2. Clinical uses

BoNT products are neuromuscular blocking agents which exert their effect through inhibition of acetylcholine release. BoNTs are amongst the most tissue-selective drugs known in clinical pharmacology and are characterised by high potency, high specificity and long duration of action of around 3–6 months following a single injection [6]. These characteristics have made BoNTs highly successful and effective therapeutic agents for the management of several chronic and debilitating diseases of neuronal hyperactivity. Although initially thought to inhibit acetylcholine release only at the neuromuscular junction, BoNTs are recognised to also inhibit release of neurotransmitters from autonomic nerve terminals, for example, in glands (e.g. in hyperhidrosis), and nociceptive neurons in pain states [6, 7].

Currently, there are four formulations of BoNTs approved by the US Food and Drug Administration (FDA) for several clinical applications (see **Table 1**). Cervical dystonia, also known as spasmodic torticollis (disorder characterised by involuntary contractions of neck and upper shoulder muscles resulting in abnormal postures and/or movement of the neck, shoulder and head and that may be associated with neck pain), is the only condition for which all four formulations are approved. Other neurological conditions include spasticity (disorder characterised by tight or stiff muscles and an inability to control those muscles), with approved formulations both for adult and paediatric populations, migraine and blepharospasm (dystonia that can cause disabling eye closure). Other non-neurological therapeutic FDA-approved uses are strabismus (eye misalignment), overactive bladder, urinary incontinence and hyperhidrosis (excessive sweating).

FDA-approved indication	Treatment population	AbobotulinumtoxinA (Dysport®)	IncobotulinumtoxinA (Xeomin®)	OnabotulinumtoxinA (Botox®)	RimabotulinumtoxinB (Myobloc®)
Cervical dystonia	Adult	Approved	Approved	Approved	Approved
Upper limb spasticity	Adult	Approved	Approved	Approved	na
Lower limb spasticity	Adult	na	na	Approved	na
Lower limb spasticity	Children ≥ 2 years of age	Approved	na	na	na
Migraine	Adult	na	na	Approved	na
Blepharospasm	≥12 years of age	na	Approved	Approved	na
Strabismus	≥ 12 years of age	na	na	Approved	na
Glabellar lines	Adult	Approved	Approved	Approved	na
Overactive bladder	Adult	na	na	Approved	na
Urinary incontinence	Adult	na	na	Approved	na
Hyperhidrosis	Adult	na	na	Approved	na

na = indication not FDA-approved.

Table 1. Food and Drug Administration (FDA)-approved indications for the use of marketed botulinum neurotoxins products [1].

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Historically, BoNT products have been considered as a single pharmacological class [8]. However, the existing BoNT products vary in the identity and amount of toxin present, their formulations, the manufacturing processes and the potency methods used to determine the strength of the products [9, 10]. As a result, the different products are not considered to be interchangeable, and their respective clinical efficacy and safety are unique to each specific product [11].

In 2016, the American Academy of Neurology (AAN) published updated guidelines for the clinical use of BoNT [12]. The 2016 AAN recommendations for BoNT use, based on evidence from clinical trials, do not fully match the FDA-approved indications or AAN's previous guidelines from 2008, which is a reflection of the expanding uses of BoNTs [8]. Multiple clinical trials are being conducted to investigate the efficacy and safety of BoNTs for various clinical conditions and, in addition, pilot studies are being conducted to test the efficacy of BoNTs for new indications [9, 13, 14]. A summary of not approved new indications for which botulinum toxins are under investigation is presented in **Table 2**.

The use of BoNTs has been extended to aesthetic applications for the reduction of facial lines. According to recent statistics, BoNT injections are now the most popular of all cosmetic procedures worldwide, both surgical and non-surgical [15], and, in the US, more than 6.6 million injections were performed in 2014 alone for aesthetic reasons [16]. There are currently three BoNT products approved by the FDA for use in glabellar lines (wrinkles that appear between

Achalasia	Dysphonia	Neuromyotonia	Rhinorrhoea and/or rhinitis
Alopecia	Endometriosis	Nystagmus	Sialorrhea
Anal fissure	Esophageal spasm	Obesity	Spasmodic dysphonia
Anismus	Exotropia, esotropia, entropion	Orbital atrophy	Stiff person syndrome
Atrial flutter	Eyelid-opening apraxia	Oscillopsia	Stuttering
Autonomic dysreflexia	Facial flushing	Osteoarthritis	Synkinesis
Benign prostatic hyperplasia	Fecal incontinence	Some forms of pain	Temporomandibular joint syndrome
Bruxism	Frey's syndrome	Palatal myoclonus	Tennis elbow
Carpal tunnel syndrome	Gastroparesis	Paratonia	Tension headache
Cleft lip repair	Gustatory sweating	Peyronie's syndrome	Tetanus
Club foot	Hemifacial spasm	Piriformis syndrome	Tremor
Constipation	Hyperlacrimation	Plantar fasciitis	Trigeminal neuralgia
Cystitis	Lateral epicondylalgia	Protective ptosis	Vaginismus
Depression	Myofascial pain	Psoriasis	Ventricular arrhythmias
Diabetic polyneuropathy	Myokymia	Restless leg syndrome	Vocal tics

Table 2. Not approved new indications for which botulinum toxins are under investigation [9, 13, 14].

the eyebrows): abobotulinumtoxinA (Dysport®, Ipsen as the marketing authorisation holder with Galderma as distributor in the aesthetic indication), incobotulinumtoxinA (Xeomin®/Bocouture®, Merz) and onabotulinumtoxinA (Botox®/Vistabel®, Allergan) (see **Table 1**). The facial aesthetic uses of BoNTs are extensive, mainly not approved and under investigation, and patient satisfaction with treatment is very high, with significant improvement in patient-reported outcomes. Rhytides (skin wrinkles) regions for treatment include forehead, brow, region between the eyebrows, around the eyes (crow's feet) and nose (bunny lines), smile (gummy smile), upper lip, corners of the mouth, jaw, chin and neck area [16, 17].

Despite intense use of BoNTs in clinical practice, approval and labelling guidance does not exist to address key questions such as where BoNTs fit amongst various treatment options for a given condition, recommendations of one product over another for a given indication or clinical differences in potency and duration of action (see Refs. [8, 18]).

3. Disease and bioterrorism threat

Botulism is a rare but potentially fatal disease caused by BoNT intoxication. Botulism is characterised by a descending flaccid paralysis with symptoms of cranial nerve dysfunction such as diplopia (double vision), dysphagia (difficulty in swallowing), pupillary dilation and ptosis (drooping eyelids), progressing to respiratory failure and, in rare occasions if not provided with suitable intensive care and life support, ultimately death. Fever and altered mental status are absent. The diagnosis of botulism is largely clinical and is confirmed by laboratory tests, sometimes including the detection of BoNTs in contaminated materials, food or bodily waste [19]. Botulism in humans is classified according to the route of entry of the toxin: food-borne botulism occurs after the ingestion of BoNT-contaminated food that contains the preformed toxin; infant botulism is the result of bacteria colonising the immature gastrointestinal tract of infants which then produce and release the toxin in situ; wound botulism results from spore contamination into the tissue and is mostly associated with injection drug abuse; and iatrogenic botulism can occur as a result of excessive BoNT use either for therapeutic or cosmetic use [20]. Inhalation botulism is also a possibility, if the toxin were to enter through the respiratory tract. However, inhalation botulism is rare and does not occur naturally [21].

A stable number of cases of botulism have been reported in Europe (i.e. European Economic Area, comprised of 31 countries) in recent years. During the period 2007–2014, an average of 115 cases per year of confirmed botulism occurred, and 5% of those were fatal [22]. A very similar numbers in the US were reported by the Centres for Disease Control and Prevention (CDC) for the same period (2007–2014), with an average of 143 confirmed cases per year, with 2% of those being fatal. According to the CDC, the most numerous cases were of infant botulism, but wound and food-borne botulisms were also presented yearly, plus a minor percentage of cases of unknown aetiology [23].

There is currently no approved pharmacological treatment for BoNT intoxication in humans, and recent efforts have focussed on the development of (1) vaccines from partially purified toxins, (2) use of specific antitoxin antibodies and (3) small molecule inhibitors [20, 24]. Once an

outbreak occurs, medical treatment includes treatment with the botulin heptavalent antitoxin and consideration of admission to an intensive care unit with mechanical ventilation until recovery. Botulism is not contagious, and standard precautions are sufficient for infection control [19].

Botulism also occurs in animals and begins with the growth of the BoNT-producing bacteria in decaying carcasses followed by the release of the toxin into the environment. Both toxin and bacteria can spread via transmission of BoNT-insensitive animals such as maggots and other invertebrates that are consumed by healthy BoNT-sensitive animals, which eventually die and allow the growth of the bacteria and the subsequent production of the toxin to self-amplify the cycle [20].

Partly due to the fact that no effective treatment is available for BoNT intoxication in humans and the perceived ease in which it could be used in a bioterror attack, BoNT is classified as a potential bioterrorism weapon by the US CDC. BoNT belongs to the category A, the highest level of concern regarding public health and need of preparedness. Only five other agents are classified as category A agents, those being anthrax (*Bacillus anthracis*), bubonic plague (*Yersinia pestis*), smallpox (*Variola major*), tularemia (*Francisella tularensis*) and arenaviruses causing viral hemorrhagic fevers [19]. A contentious paper from 2005 regarding ease of BoNT intoxication through cow's milk destined for human consumption calculated if would take only 4 g of BoNT, e.g. roughly equivalent to 1 teaspoon of granulated sugar, to poison over 400,000 people [25]. The publication of that research opened a public safety debate within the scientific community regarding BoNT dual-use research [26], which was reopened when the allegedly new BoNT/H type was originally reported [27]. However, it should be noted that BoNTs are much more toxic (in the range of 100–1000 times) when injected than when administered orally; and delivery by aerosols is considered inefficient [20].

4. Historical overview of BoNT discovery

BoNT/A is the most potent toxin known to man, with a reported estimated human lethal dose of 1.3–2.1 ng/kg intravenously or intramuscularly and 10–13 ng/kg when inhaled [4]. Not surprisingly, its effects have been known throughout history long before the molecular identity of the toxin was elucidated. Botulism-like symptoms were known by ancient Greeks and Egyptians, and the Byzantine emperor Leo IV (886–911 AD) banned 'blood sausage' as it caused a fatal illness. It was not until around a thousand years later that following a number of sausage poisoning outbreaks in Germany the first accurate and complete description of the symptoms of food-borne botulism was described between 1817 and 1822 by J. Kerner. The extracted causative agent was named 'sausage poison' and was believed to be a 'fatty acid'. Later, a German physician named Muller referred to the sausage poisoning as botulism from the Latin name for sausage, 'botulus' [4, 28].

The first isolation of the bacteria responsible for producing the toxic agent causing botulism was performed by the Belgian professor Emile Pierre van Ermengem and was termed *Bacillus botulinus*. Its name was changed to *Clostridium botulinum* when the aerobic *Bacillus* genus was separated from the anaerobic *Clostridium* genus [29]. To date, six different BoNT-producing bacterial groups are known; all have been taxonomically classified as clostridia. These clostridia

produce seven different serotypes of botulinum toxin, termed BoNT/A to BoNT/G. BoNT/A, BoNT/B and BoNT/F were discovered following incidences of food-borne botulism, reminiscent of the original 'sausage poisoning', whereas BoNT/C, BoNT/D and BoNT/E were discovered following incidences of botulism in animals [27] (see **Figure 1**). BoNT/G, discovered in 1970, was reported in a sample extracted from soil, and to date there has not been reported cases of botulism caused by BoNT/G in the wild affecting either humans or animals [30]. A possible eighth type, initially termed BoNT/H was reported in 2013, but later reclassified as a BoNT/FA hybrid [31].

Despite Kerner suggesting the potential of BoNT as a therapeutic agent in conditions of muscular hypercontraction and glandular hypersecretion, it was not until around 150 years later

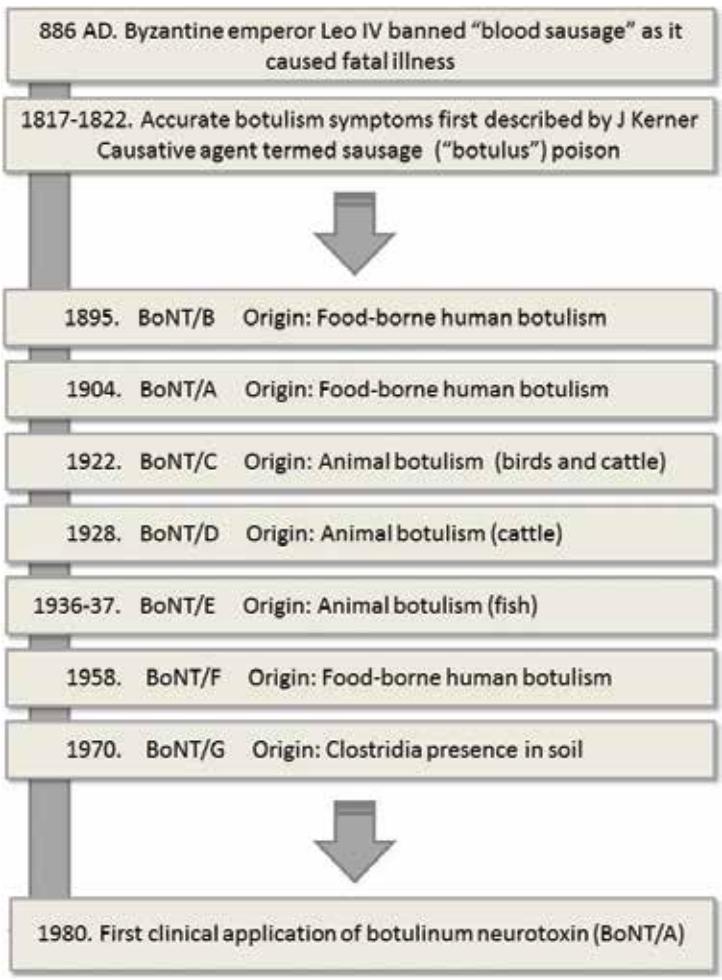

Figure 1. Timeline of the discovery of the seven botulinum toxin types. For context, also depicted are the dates of the first accurate description of botulism and the first use of botulinum toxins as therapeutic agent. A proposed eighth type was initially reported in 2013, now classified as an F/A hybrid toxin. For details see Refs. [2, 27, 28].

that the first clinical application was made. In 1981, Dr. Alan B. Scott at what was formerly known as the Smith-Kettlewell Institute of Visual Science, San Francisco, California, USA, used BoNT/A for the treatment of strabismus as an alternative to surgical intervention. The original name of the drug was Oculinum®, and its rights were later acquired by Allergan Inc., which changed the name of the drug to Botox®.

5. The producing bacteria: types of toxin and nontoxin proteins

Upon the first description of the botulism symptoms (see above), the initial hypothesis was that botulism was caused by a toxin produced by a single bacterial organism, as is the case for the closely related toxin tetanus toxin and its producing bacteria *Clostridium tetani* [32]. However, it soon became apparent that different types of toxin and different producing bacteria existed for BoNT [29].

In 1910–1919, serological methods were introduced for categorisation of the toxin-producing bacteria and for the toxins themselves, that are still in use today [33]. Biochemical and molecular techniques have complemented those initial classifications and have confirmed the presence of multiple species of BoNT-producing clostridia and multiple species of BoNT proteins. BoNT-producing bacteria are Gram-positive, anaerobic, spore-forming and rod-shaped organisms and are commonly found in any soil or water environment. The seven distinct serotypes differ by 37–70% in amino acid sequence [34]. Early observations pointed to a level of intratypic serological diversity that led to variants within serotypes to be called sub(sero)types and a proposal that new subtypes would differ by 2.6% at the protein sequence level. However, this rule is not consistently applied today throughout all the subtypes [35]. It is considered that 41 individual toxins exist and the various toxin subtypes are given a letter designation for the toxin serotype followed by a sequential number in order of discovery, e.g. BoNT/A1 and BoNT/E11. Only 4 serotypes currently present subserotypes, namely BoNT/A (8 subtypes), BoNT/B (8 subtypes), BoNT/E (12 subtypes) and BoNT/F (7 subtypes) (see **Figure 2**). Interestingly, BoNT/C and BoNT/D occur naturally as well as hybrid toxins, termed BoNT/CD and BoNT/DC. A third naturally occurring hybrid, BoNT/FA, was initially proposed as the new serotype BoNT/H following its discovery in 2013 but later reclassified as a hybrid toxin [36].

Current classification of BoNT-producing clostridia is according to group designation based on metabolic biochemical criteria (see **Table 3**). The metabolic groups represent distinct species of *Clostridium botulinum* (Groups I to III) and *Clostridium argentinense* (Group IV), and these species include non-toxigenic as well as neurotoxigenic members. In addition, *Clostridium baratii* and *Clostridium butyricum* are also known to produce BoNTs (Groups V and VI). To add to the confusion, some *Clostridium botulinum* strains do not produce BoNT, in particular if subcultured repeatedly in the laboratory; and some additional toxins are produced by the neurotoxigenic *Clostridium botulinum*, such as C2 toxin, C3 exoenzyme and botulinolysin. However, no alternative nomenclature for this group of organisms has been accepted [32].

Clostridial strains in different groups can produce the same toxin (e.g. Groups I, II and V produce BoNT/F), and bivalent toxin combinations within the same strain have been identified.

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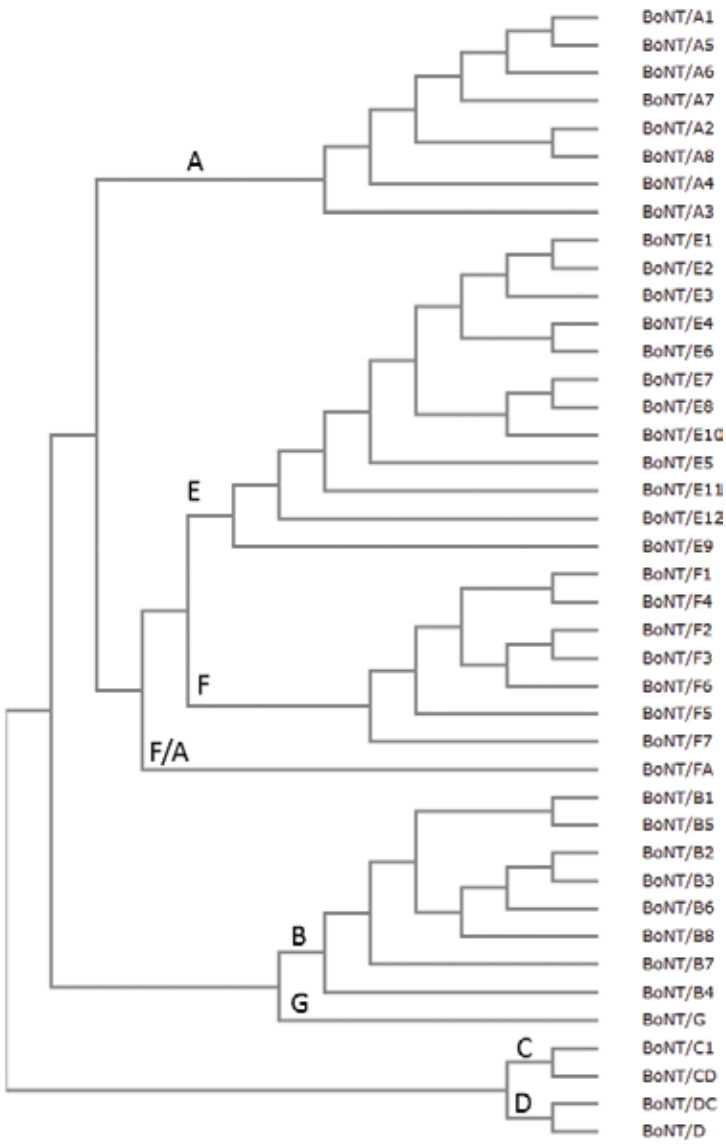

Figure 2. Phylogenetic tree depicting the relationship of the 41 known botulinum neurotoxins. Individual FASTA files were accessed through the NCBI portal (NIH, USA), and the protein alignment and phylogram were constructed using the online software Clustal Omega (EMBL-EBI, Germany).

When more than one toxin is produced by a single strain, such as Ba or Bf, the capital letter designates the toxin produced in greater amounts. If a gene is present but not expressed, it is denoted between brackets, for example, A(B); and if a gene is present but truncated, it will have an apostrophe to indicate this fact, such as A(B'). This diversity in BoNT-producing bacterial strains is the result of toxin gene associations with transposases such as insertion sequence elements, recombinases, the acquisition of plasmids or infection by phage [37], within and between the

Clostridial bacteria	Group	BoNT serotype(s) produced	Mixture of serotype(s) produced by a single strain	Nontoxinogenic bacteria belonging to the same group
Clostridium botulinum	I	A, B, F	A(B), A(B'), Ab, Af, Ba, Bf, Bf/a	*Clostridium sporogenes*
Clostridium botulinum	II	B, E, F	–	*Clostridium taeniosporum*
Clostridium botulinum	III	C, D, CD hybrids DC hybrids	–	*Clostridium novyi*
Clostridium argentinense	IV	G	–	*Clostridium argentinense* *Clostridium subterminale* *Clostridium hastiforme*
Clostridium baratii	V	F	–	*Clostridium baratii*
Clostridium butyricum	VI	E	–	*Clostridium butyricum*

Table 3. BoNT-producing clostridial species, see Refs. [20, 29, 38].

groups and species. Groups IV–VI have the toxin genes located in the chromosome, considered less mobile, whereas Group III has the toxin genes in highly mobile elements such as plasmids and bacteriophages. Groups I and II have a mixture of chromosome and plasmid localisation [38]. A recent genetic study of *C. botulinum* strains causing human botulism in France showed that the genetic diversity of the BoNT-producing organism appeared as a result of multiple and independent genetic rearrangements and not from a single evolutionary lineage [39].

All seven BoNT serotype toxins are released from the producing bacteria as large protein complexes with a number of neurotoxin-associated proteins (NAPs) to become highly potent oral toxins, often ingested in contaminated foods [38, 40]. The NAPs are encoded together with the *bont* gene in one of two different gene clusters, the hemagglutinin (HA) cluster or the orfX cluster. Both clusters encode the nontoxic non-hemagglutinin (NTNHA) protein, which assembles with BoNT to form the smaller of the progenitor toxin complexes. BoNT/A, BoNT/B, BoNT/C and BoNT/D complexes contain HA, whereas BoNT/E and BoNT/F complexes do not contain HA. The components of the BoNT complex vary with neurotoxin serotypes and the *Clostridium* strain producing them. BoNTs are produced in three progenitor forms: M (medium), L (large) and LL (extralarge) complex. The M form consists of the neurotoxin (of 150 KDa) with NTNHA and has a total weight of ~ 300 KDa. The L and LL complexes consist of several HA proteins besides the BoNT and NTNHA, and its molecular weight is ~ 500 KDa for the L form and ~ 900 KDa for the LL form. The function of the proteins encoded in the orfX genes remains unknown [41]. NAPs are known to protect BoNTs against the proteases of the gastrointestinal tract and the acidic conditions of the stomach and to facilitate the intestinal trans-epithelial delivery to the toxin into the lymphoid and general circulation [38]. The role of NAPs in the producing bacteria is not known. Recently, it has been proposed that the primary role of NAPs and in particular that of NTNHA is to protect BoNTs from damage in the decaying biological material where the toxin is mostly produced in the wild [20].

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Until recently, BoNTs were believed to be produced exclusively by clostridia organisms. In 2015, the first homologue of BoNTs was described within the genome of the rice fermentation bacteria *Weissella oryzae* SG25 [42]. Bioinformatic analysis of the genomic sequences of *W. oryzae* SG25 revealed one gene with a very similar structure to BoNTs, whereas a second gene showed partial similarity with the BoNT-associated NTNH proteins [42]. Recombinant expression of the BoNT-like protein revealed that it shares similarities with BoNT/B regarding its targeting profile and it is also expected to block neurotransmitter release. The new BoNT-like protein showed no serological cross-reactivity with the seven known BoNT serotypes, and it was dubbed BoNT/Wo by the authors [43].

6. Structure-function of BoNT toxins

BoNTs are zinc metalloproteases consisting of three major domains. Produced as a single polypeptide of 150 KDa, BoNTs require activation by cleavage of the polypeptide post-translationally resulting in the so termed heavy chain, of ~ 100 KDa, and a light chain (LC), of ~ 50 KDa, held together by a disulphide bridge between the two chains [44]. Functionally, the light chain hosts the metalloprotease domain, and the heavy chain comprises both the binding domain (H_C) and the translocation domain (HN). The producing bacteria in Groups I, III and IV (see **Table 3**) are proteolytic strains and will release the cleaved active product, whereas the products of the other producing bacteria are believed to be activated by proteases of the intoxicated organism [38].

The neuromuscular junction is the natural target of BoNTs, and intoxication follows an intricate multistep mechanism [20, 45], in which the toxin-associated proteins of the progenitor toxin complex play a crucial role. For an overview of the routes of entry and mechanism of action of the toxin, see **Figure 3**.

Unintentional BoNT entry into the organism occurs mainly through ingestion of contaminated foods leading to food-borne botulism (see above) or through wounds [20]. Alternatively, and in particular in cases of infant botulism, the producing bacteria can colonise the immature gastrointestinal tract and produce the exotoxin in situ. The progenitor complex allows BoNTs to effectively cross the intestinal trans-epithelial barrier and reach the lymphoid and general blood circulation. Under neutral and alkaline environments, such as in the bloodstream, the complex dissociates and the naked toxin is able to target neuromuscular junctions [46]. In clinical applications, the toxin is delivered locally to the site of action. BoNT entering the body undergoes a relatively short distribution phase which sees the toxin selectively targeting peripheral nerve endings, and an elimination phase that comprises both (1) an interneuronal metabolism following cellular entry and (2) systemic metabolism and elimination which are assumed to be through the liver [47].

Upon reaching the neuromuscular junction, BoNTs are able to specifically target nerve terminals using their Hc-binding domain and internalise through endocytosis. Once in the acidic environment of the endosome, the BoNT HN domain translocates the LC domain into the cytosol, allowing the Zn^{+2} metalloprotease enzyme to cleave target soluble N-ethylmaleimide-sensitive

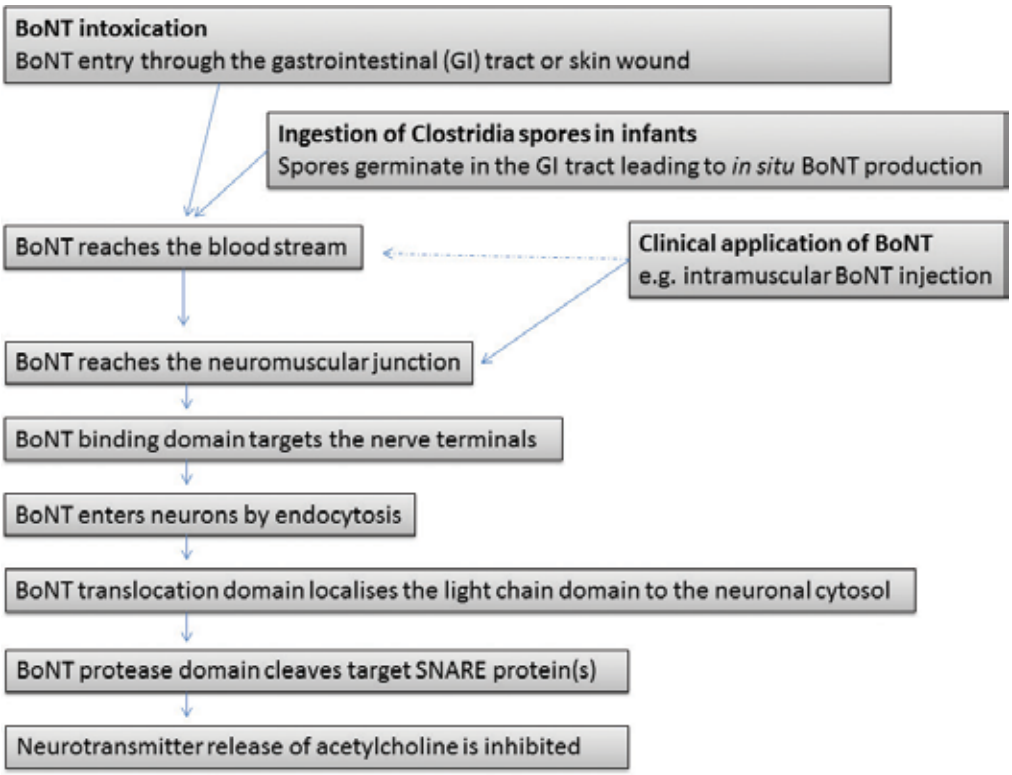

Figure 3. Mode of action of botulinum toxins; for details in each step, see Refs. [20, 45].

factor attachment protein receptor (SNARE) proteins. SNARE proteins constitute an essential part of the machinery for neurotransmitter release in eukaryotic cells, and once their function is compromised by BoNTs, release of acetylcholine in the neuromuscular junction is prevented [19].

Although all serotypes, and even the most recently described BoNT-like protein BoNT/Wo [43], share a multidomain structure, crystallographic data has revealed that the molecular arrangement in the 3D space varies. BoNT/A and BoNT/B present an 'open butterfly' structure, whereas BoNT/E has a 'closed butterfly' organisation when viewed taking the HN translocation domain as a sagittal axis [48, 49]. In **Figure 4**, three different representations illustrate the organisation of BoNT/A and BoNT/E. This differential 3D topology has been credited to confer particular characteristics to BoNT serotypes, such as a faster way of entry for BoNT/E compared to BoNT/A [50].

6.1. Binding domain

BoNTs belong to the family of AB exotoxins, consisting of an 'A' toxic domain and a 'B' binding domain. AB toxins such as cholera toxin, lethal factor from *Escherichia coli* and Shiga toxin use gangliosides as their cellular receptors; whereas anthrax toxin and ricin have protein receptors identified as their targets [51].

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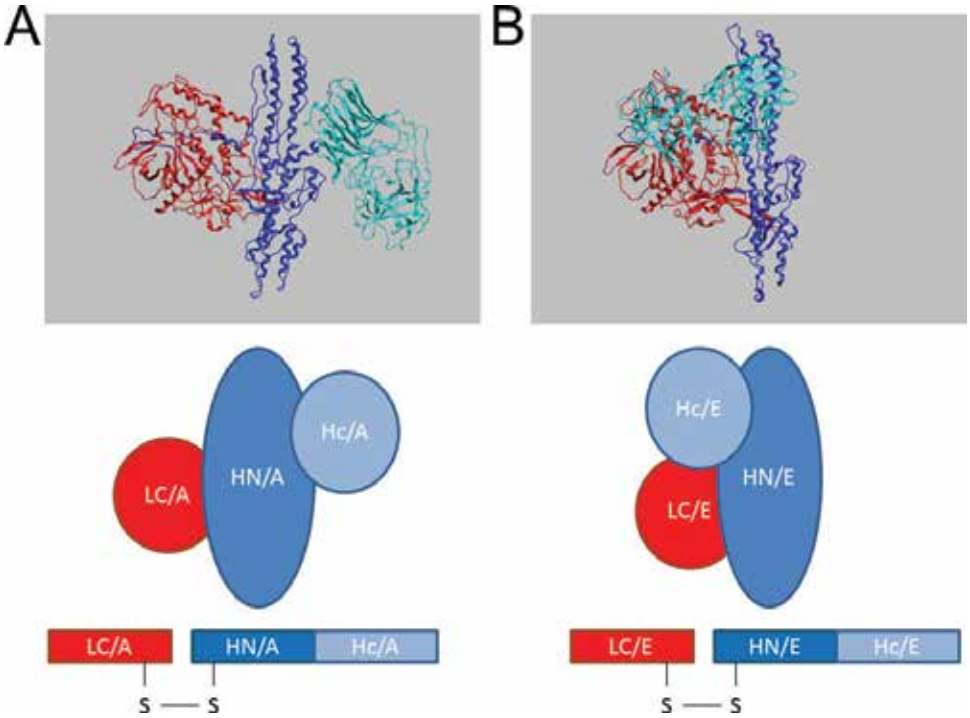

Figure 4. Structural and functional domains of (A) BoNT/A (PDB 3BTA) and (B) BoNT/E (PDB 3FFZ). Hc = binding domain, HN = translocation domain, LC = light chain protease domain. Upper panels: ribbon diagram of the respective crystal structures. Middle panels: diagram depicting the three-dimensional organisation of the domains within the structure. Lower panels: simplified two-dimensional block diagram in which the HN and LC can be seen being connected by a conserved disulphide bridge. Structural image created from crystallographic using the MOE software (Molecular Operating Environment 2013.08; Chemical Computing Group Inc., Montreal, Canada).

In the case of BoNTs, a dual receptor theory was postulated [52]. This dual-binding anchorage is credited for the high affinity and specificity by which BoNTs target neurons. All serotypes share a similar binding site for the interaction with the oligosaccharide portion of a polysialoganglioside. For BoNT/A, BoNT/B, BoNT/E, BoNT/F and BoNT/G, the conserved ganglioside-binding site SXWY has been reported, whereas BoNT/C, BoNT/D and BoNT/DC have analogous sites for ganglioside binding at a similar position [53]. A second, non-conserved binding site that binds a protein receptor has been identified in several BoNTs [54]. BoNT/A, BoNT/E and BoNT/F bind the family of synaptic vesicle protein SV2, whereas BoNT/B, BoNT/D and BoNT/G recognise a short peptide sequence in the luminal domain of the family of synaptic vesicle protein synaptotagmin. A protein receptor has not yet been identified for BoNT/C, which uses a dual ganglioside mechanism [55]. A second protein receptor has been identified for BoNT/A, namely, FGFR3 [56]. Crystal structures of the H_C domains in complex with their receptors, where available, have contributed a major advance in the understanding of BoNT-cell interactions.

Recently, glycan motifs in both gangliosides [53] and protein receptors [57, 58] have emerged as key players in the targeting of BoNTs to the neuronal membranes, albeit glycosylation is not required for binding for all BoNTs [38].

6.2. Translocation domain

Neuronal internalisation triggers the translocation of the LC domain to the cytosol, separating it from the HN and H_C domains and thus allowing it to cleave the cytosolic target SNARE proteins. In neurons in vitro, internalisation of BoNT/A and translocation of the LC into the cytosol occur rapidly, with estimates either side of ~ 60 minutes [59]. Following entry into the synaptic vesicle, the proton pumping action of the v-ATPase present on the synaptic vesicle membrane, responsible for the loading of neurotransmitters into the vesicle, will acidify the organelle and produce the necessary environment for the LC to translocate. Treatments that inhibit internalisation, synaptic vesicle recycling, or acidification also inhibit BoNT action [60].

Of the various steps of the cellular mechanism of intoxication, membrane translocation for the LC is least understood at the molecular level, and several models have been suggested [48]. The first model proposes that upon acidification of the lumen of the synaptic vesicle, HN penetrates the membrane and forms an ion channel assisting a partially unfolded LC to pass through it. This model has been revised and a new proposed mechanism includes binding of the toxin domains to the luminal membrane of the synaptic vesicle, and following acidification both HN and a partly unfolded LC will destabilise and penetrate the membrane. LC will move to the cytosolic side, refold and be released upon reduction of the disulphide bond. At the same time, segments of the HN insert in the membrane and assemble an ion channel. The main difference between these models is that in the first model, channel formation by HN is an early event and translocates LC, whereas in the second model, the channel formed by HN occurs as a consequence of the LC translocation. In both models, the reduction of the disulphide bond is essential to free the LC at the end of the translocation step, and the enzyme thioredoxin and its regenerating enzyme thioredoxin reductase have been identified as the cellular system responsible for the reduction of the disulphide bridge [61]. Following translocation, another key protein recently identified is Hsp 90, which may act as a chaperone assisting the refolding of the LC once in the cytosol [62].

Different models have been proposed for the mechanism by which BoNT domains approach the membrane, which may have physiological consequences. BoNT/E is thought to owe its rapid translocation to its 'closed butterfly' three-dimensional structure in which the Hc and LC are in close proximity [50], whereas BoNT/A and BoNT/B, which in principle share the 'open butterfly' configuration, would approach the membrane differently [63].

6.3. Protease domain

The LC domain is a metalloprotease that cleaves SNARE proteins within the nerve terminal cytosol, resulting in the inhibition of the acetylcholine release which causes a reversible neuroparalysis [44].

SNARE proteins are membrane-associated proteins and comprise a large family of proteins that are responsible for the binding and fusion of vesicles to membranes. In humans, there are 38 different types of SNARE proteins [64]. SNARE proteins that mediate the exocytosis of neurotransmitter vesicles with the plasma membrane of neurons are the target substrates of BoNTs [65]. In addition to inhibiting neurotransmitter release, SNARE cleavage by BoNT also

affects trafficking of proteins, for example, TRPV1 and TRPA1 receptors to the neuronal surface [66]. BoNT/A, BoNT/C and BoNT/E target SNAP-25, whereas BoNT/B, BoNT/D, BoNT/F and BoNT/G target VAMP1, VAMP2 and VAMP3 proteins. BoNT/C is unique amongst BoNTs in targeting two different SNARE types, as it targets syntaxin 1 and syntaxin 2 besides also targeting SNAP-25. Hydrolysis of the SNARE proteins occurs at a unique cleavage site specific to each BoNT [35].

No additional target substrates have been reported for BoNTs beyond SNARE proteins. This may be due to the extensive interaction that BoNTs make with the target proteins, including the cleavage site, which may be responsible for the exclusive specificities to SNARE isoforms in a species-specific manner [48].

The length of BoNT-induced intoxication may depend on (1) how long the cleaved SNARE proteins remain in the cytosol and the ability of the cleaved SNARE proteins to maintain the block to exocytosis, (2) how long the BoNT protease remains in place to cleave newly synthesised SNARE proteins, (3) the rate at which the neuron is able to replenish uncleaved SNARE proteins relative to ongoing cleavage, and (4) the ability of the presynaptic terminal to remodel in order to overcome the temporary paralysis. There is preclinical evidence for all these hypotheses [67]. The ubiquitination pathway has been proposed as a main mechanism responsible for degradation of the LC in the cytosol, thus terminating BoNT activity [68].

6.4. Three domains and four functions

Despite intense activity in recent years towards understanding the basic mechanism of action (MOA) of BoNTs, currently known structure-activity relationships of the four BoNT functions (binding, internalisation, translocation and SNARE cleavage at the nerve terminals of the neuromuscular junction) within three domains (Hc, HN, and LC) are not fully understood. Current gaps in basic understanding include molecular details of the specificity of the binding of each BoNT to neurons, entry into the nerve terminal and translocation of the LC, the correlation between SNARE cleavage and neuroparalysis and the length of BoNT-induced neuroparalysis. For example, it is known that the length of paralysis varies with BoNT type, dose, animal species and type of nerve terminal (from 3 to 4 months for skeletal nerve terminals to 12–15 months for autonomic cholinergic nerve terminals) [54, 69]. Furthermore, there are emerging functions that do not fall within the canonical intoxication pathway.

Regarding discrete functions of BoNT domains, there is increasing evidence that, in addition to their individual functions, each domain influences the other to work in concert to achieve BoNT intoxication. For example, the binding domain is not necessary for cell entry or LC translocation, but it determines the pH threshold for HN channel formation during the translocation step [70].

Entry of BoNTs has also been reported independent of synaptic vesicle recycling [71]; and retrograde transport within non-acidifying organelles, a characteristic of the related tetanus toxin, has been described for BoNT/A and BoNT/E [72]. Effects of BoNT in the central nervous system, such as in pain states, have also been reported, indicating actions beyond the neuromuscular junction that would involve retrograde transport of the toxin [73].

Furthermore, BoNTs, and in particular BoNT/A, are known to exert further actions unrelated to the cleavage of SNAP-25, at doses/concentrations that prevent SNAP-25-mediated neurotransmitter release. These activities include (1) increasing the proteosomal degradation of the protein RhoB in arachidonic-mediated neuroexocytosis, (2) induction of neuritogenesis, (3) reduction of cellular proliferation and (4) effects on gene expression, both in in vivo and in vitro settings [74]. The significance of these findings is not yet fully understood, but opens exciting opportunities to expand the use of BoNTs beyond their classical SNARE-cleaving MOA.

7. New therapeutics

7.1. Improvements on current products

The four FDA-approved formulations in the market for BoNT products are manufactured starting with the fermentation of the respective *C. botulinum* [1]. As a result, the manufacturing processes come with their own challenges, namely, (1) the anaerobic requirements mean that oxygen must be excluded from the first stages of the production system as the *C. botulinum* are obligate anaerobes, (2) the production of the toxin progresses from the first stages of growth, so health and safety measures are paramount throughout the manufacturing process, (3) sporulation of the bacteria can occur at low levels during the growth stages, but particularly when the bacterial life cycle ends and the bacteria die, and (4) the nutritional growth requirements of *C. botulinum* are not known in detail, which results in complex growth media adding extra degrees of complexity [75]. Recombinant production of BoNTs in non-obligate anaerobes and non-sporulating organisms, already widely used in the research setting (e.g. Ref. [76]), will simplify the manufacturing process enormously, as well as facilitate molecular engineering approaches that are state of the art in the protein field.

One aspect that is still contentious about the current BoNT drug products is the presence/absence of the ancillary non-toxic associated proteins (NAPs). In particular, it is not clear what role these proteins, which are critical to protect the toxin during entry through the gastrointestinal tract, are playing when the toxin is injected, as is the case for the current therapeutic and aesthetic uses. AbobotulinumtoxinA (Dysport®) and onabotulinumtoxinA (Botox®) present a complex of BoNT plus non-toxic associated proteins (NAPs), whereas incobotulinumtoxinA (Xeomin®) does not have NAPs present in its formulation [11]. RimabotulinumtoxinB (Myobloc®) is also a neurotoxin complex in which the BoNT is associated with hemagglutinin and non-hemagglutinin proteins [1].

Regarding distant spread, the FDA prompted an inclusion of a black box warning for all FDA-approved BoNT products, as follows: 'The effect of all botulinum toxin products may spread from the area of injection to produce symptoms consistent with botulinum toxin effects. These symptoms have reported hours to weeks after injection. Swallowing and breathing difficulties can be life-threatening and there have been reports of death' [1]. Ancillary proteins are not likely to play a role in distant spread since studies show that there were no differences in product diffusion when the same dose was injected with the same technique [15].

Triggering of immune responses by BoNT use, and possibly triggering non-responsiveness to treatment, is a controversial topic since, despite dissociation from the toxin NAPs, HA and NTNHA proteins form part of the protein load of the injection [77]. Following meta-analysis of clinical incidence of neutralising antibody immunogenicity is often revealed as a very minor issue with low, single-digit percent occurrence with the current main products [78]. Differences have been seen with an older product (which exhibited higher incidence of neutralising antibodies), dosing frequency and cumulative dose [79].

New products, produced using different manufacturing processes and with different final formulations, may help address the above issues and indeed as well for the existing natural products, which have not changed formulation or manufacturing process significantly in the last 20 years [75]. Alternative new products include Nabota® (Daewoong Pharmaceutical Co., Korea), which consists of BoNT/A obtained following a special purification process, and RT002 (Revance Therapeutics Inc., USA), which is an injectable formulation of BoNT/A containing a polycationic excipient developed to limit diffusion of the toxin into adjacent tissues and to be longer acting than the current BoNT products, amongst others [2, 9]. The use of hydrogels and liposomes, for example, in treatments for bladder or gastric disorders, has also been reported as novel BoNT formulations being investigated [80]. Liquid formulations for BoNT/A products, already in the market for BoNT/B, are actively being pursued, and their use would preclude the need of reconstitution of the products [75].

7.2. Molecular engineering of BoNTs

Given the natural diversity of BoNTs, with 7 serotypes and over 40 individual subtype proteins, it is surprising that the leading marketed products are restricted to only two serotypes, BoNT/A and BoNT/B. So far, anecdotal use of BoNT/C and BoNT/F was reported few years ago [81]. The current landscape of new therapeutics include, for example, the potential use of the short-acting BoNT/E1 as reported in WO2014068317 [82], the use of a BoNT/B toxin with increased binding affinity for its human cognate receptor synaptotagmin II as reported in WO2013180799 [83] as well as BoNT/A3 (WO2013049139 [84]). In particular, serotype BoNT/A2 has been extensively studied in Japan as an alternative BoNT/A with differentiated biology [85, 86].

Molecular engineering approaches facilitate the harnessing of inherent characteristics present in the already diverse natural BoNTs [87] but also allowing the introduction of new properties. When considering engineering approaches, all three BoNT domains offer exciting opportunities; for a recent review, see Ref. [5]. Firstly, engineering of the Hc domain could facilitate (1) alternative receptor targets to modify specificity, (2) allow immune epitope modification and (3) add/modify receptor-binding motifs and related structural regions to modify affinity. Secondly, engineering the HN domain could modulate cargo capacity and pH dependency of translocation of cargo. Finally, LC engineering could provide (1) substrate specificity, (2) desired intracellular localisation, (3) modification of immune epitopes and (4) modification/manipulation of self-proteolysis and degradative pathways.

An example of such engineering approaches is targeted secretion inhibitors (TSI), in which the Hc domain of BoNT is substituted by an alternative cellular targeting domain (e.g. see WO2006059093 [88]), which will be discussed in the next section.

7.3. Example of new therapeutics: targeted secretion inhibitors

Natural BoNT toxins target neuronal terminals, and their duration of action is often measured in months. These characteristics have made them very successful therapeutic and aesthetic agents (see Section 1 above), but it also limits their use to their specific target cells. Given that SNARE proteins underpin a universal mechanism of secretion in eukaryotic cells, an engineering approach that would lead to cleaved SNARE proteins in a wide range of (hypersecreting) cells would provide novel and exciting therapeutic opportunities. In TSI, the Hc-binding domain of BoNTs is substituted by an alternative cell-binding moiety, and the resulting proteins are not neurotoxins but a new class of biopharmaceuticals [89].

The basis for the TSI platform development is a functional fragment from BoNTs comprising the LC and HN domain, termed LHN. LHN proteins are proteolytically cleaved during activation, and the two domains remain connected by a disulphide bridge, as is the case in the parental BoNTs. LHN/A, LHN/B, LHN/C and LHN/D are amenable to recombinant expression in *E. coli* and have all been described as functionally active, resembling the respective parental toxin [90, 91]. Examples of TSI include those where the targeting domain is comprised of wheat germ agglutinin, nerve growth factor, an epidermal growth factor receptor (EGFR) targeting ligand or a growth hormone-releasing hormone receptor (GHRHR) targeting ligand. These TSI have shown that it is possible to achieve internalisation of the active BoNT LC contained in their structure into non-neuronal cells otherwise resistant to the parental BoNT [92–94].

The structures of LHN/D and a GHRHR-targeted TSI/D, SXN101959, are shown in **Figure 5**. When compared with BoNT structures depicted in **Figure 3**, it is seen that the BoNT Hc domain is absent in the LHN structure and, in the case of the TSI a new targeting moiety takes the place of Hc. Often, the new targeting moiety is considerably smaller than the original Hc domain of the parental BoNT. That poses its own challenges regarding ligand accessibility, and so linkers and spacers are frequently used. Furthermore, in the case of this GHRHR ligand, a free N-terminus of the peptide is required for optimal activation of the GHRHR receptor [95], which has prompted the position of the ligand to be at the N-terminal end of HN when compared to the Hc (located at the C-terminal of HN in the natural structure). Functionally, this TSI has been shown to exert a powerful and reversible inhibitory action on the endocrine growth hormone and insulin-like growth factor-I axis [96].

Little is known about TSI intracellular trafficking, and it is generally assumed that the BoNT four-step MOA (binding, internalisation, translocation and SNARE cleavage) will apply. A study using a GHRHR-targeted TSI/D reported an intracellular, punctate, immune-staining pattern indicative of the presence of the TSI in endosomes [97]. In a recent paper, internalisation of an EGFR-targeted TSI/A and BoNT/A was assessed in the same cellular system [98]. The EGFR-targeted TSI/A partially internalised in an intracellular compartment consistent with endosomes, whereas BoNT/A did so in a different compartment consistent with synaptic vesicle recycling. Both proteins were able to cleave the cytosolic SNARE protein target SNAP-25. The study confirmed that BoNT domains are a versatile tool to extend the pharmacological effect of BoNTs beyond the natural target of the neuromuscular junction.

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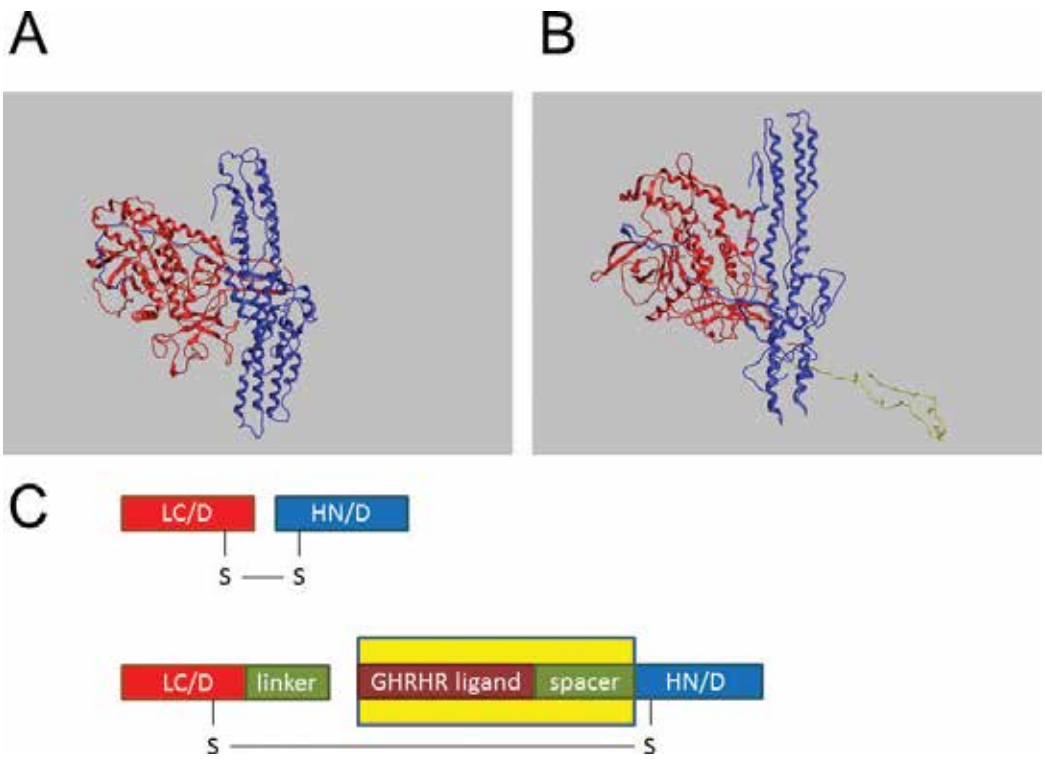

Figure 5. Structural and functional domains of LHN/D and a GHRHR-targeted TSI/D. (A) Crystallographic data of LHN/D (PDB 5BQN). (B) Crystallographic data for the GHRH-targeted TSI/D (PDB 5BQM) in which the targeting domain has been added using molecular modelling for illustration purposes. The ribbon to the right of the LC and HN domains corresponds to the GHRHR ligand plus the spacer, as illustrated in (C). (C) Simplified block diagrams of the structures presented in (A) and (B), respectively. The HN and LC domains in both structures can be seen being connected by a conserved disulphide bridge. Structural images created using the MOE software (Molecular Operating Environment 2013.08; Chemical Computing Group Inc., Montreal, Canada).

In addition to the delivery of SNARE cleaving activity into non-neuronal cells, TSI can also be used to provide alternative targeting to neurons with improved neuronal selectivity. One such example is neuronal targeting *via* the nociceptin receptor, which reached Phase II clinical trials for post-herpetic neuralgia and overactive bladder (WO2006059093) [88, 99].

8. Conclusions

BoNTs are key therapeutic agents with a seemingly ever-increasing list of new applications. The fascinating modular molecular architecture and natural diversity of BoNTs is the base for future therapeutics, being developed using recombinant technologies, new formulations and engineered new pharmacological properties.

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Appendices and nomenclatures

AAN	American Academy of Neurology
BoNT	Botulinum neurotoxin
CDC	Centres for Disease Control and Prevention, US
EGF	Epidermal growth factor
EMA	European Medicines Agency
FDA	Food and Drug Administration, US
GHRHR	Growth hormone-releasing hormone receptor
HA	Hemagglutinin
Hc	BoNT-binding domain
HN	BoNT translocation domain
LC	BoNT enzymatic domain
MOA	Mode of action
NAPs	Neurotoxin-associated proteins
NTNHA	Nontoxic non-hemagglutinin protein
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins
TSI	Targeted secretion inhibitors

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References

[1] Drugs@FDA: FDA Approved Drug Products. http://www.accessdata.fda.gov/scripts/cder/daf/index.cfm [Accessed 25-May-2017]

[2] Dressler D. Botulinum toxin drugs: Brief history and outlook. Journal of Neural Transmission Vienna Austria 1996. 2016;**123**:277-279

[3] Botulinum Toxin Market Trends. http://www.strategyr.com/MarketResearch/ViewInfoGraphNew.asp?code=MCP-1833. [Accessed 25-May-2017]

[4] Foster KA. Overview and history of botulinum neurotoxin clinical exploitation. In: Foster KA, editors. Clinical Applications of Botulinum Neurotoxin. New York: Springer Science +Business Media; 2014. pp. 1-5

[5] Chaddock JA. Future developments: Engineering the neurotoxin. In: Foster KA, editors. Clinical Applications of Botulinum Neurotoxin. New York: Springer Science +Business Media; 2014. pp. 177-192

[6] Chen JJ, Dashtipour K. Abo-, inco-, ona-, and rima-botulinum toxins in clinical therapy: A primer. Pharmacotherapy. 2013;**33**:304-318

[7] Orsini M, Leite MAA, Chung TM, et al. Botulinum neurotoxin type A in neurology: Update. Neurology International. 2015;**7**:5886

[8] Albanese A. Clinical guidelines: No more mistaken identities for botulinum neurotoxins. Nature Reviews. Neurology. 2016;**12**:373-374

[9] Dashtipour K, Pedouim F. Botulinum toxin: Preparations for clinical use, immunogenicity, side effects, and safety profile. Seminars in Neurology. 2016;**36**:29-33

[10] Lanoue J, Dong J, Do T, et al. An update on neurotoxin products and administration methods. Cutis. 2016;**98**:163;166;197

[11] Brin MF, James C, Maltman J. Botulinum toxin type A products are not interchangeable: A review of the evidence. Biologics: Targets and Therapy. 2014;**8**:227-241

[12] Simpson DM, Hallett M, Ashman EJ, et al. Practice guideline update summary: Botulinum neurotoxin for the treatment of blepharospasm, cervical dystonia, adult spasticity, and headache Report of the Guideline Development Subcommittee of the American Academy of Neurology. Neurology. 2016;**86**:1818-1826

[13] Therapeutic Uses of Botulinum Toxin. Grant Cooper | Springer. http://www.springer.com/la/book/9781588299147. [Accessed 25-May-2017]

[14] Lim ECH, Seet RCS. Use of botulinum toxin in the neurology clinic. Nature Reviews. Neurology. 2010;**6**:624-636

[15] Frevert J. Pharmaceutical, biological, and clinical properties of botulinum neurotoxin type A products. Drugs RD. 2015;**15**:1-9

[16] Gart MS, Gutowski KA. Overview of botulinum toxins for aesthetic uses. Clinics in Plastic Surgery. 2016;**43**:459-471

[17] Gendler E, Nagler A. Aesthetic use of BoNT: Options and outcomes. Toxicon Official Journl of International Society on Toxinology. 2015;**107**:120-128

[18] Barbano RL. Botulinum toxins in clinical practice: Gaps in knowledge. Neurology: Clinical Practice. 2016;**6**:206-208

[19] Adalja AA, Toner E, Inglesby TV. Clinical management of potential bioterrorism-related conditions. The New England Journal of Medicine. 2015;**372**:954-962

[20] Rossetto O, Pirazzini M, Montecucco C. Botulinum neurotoxins: Genetic, structural and mechanistic insights. Nature Reviews. Microbiology. 2014;**12**:535-549

[21] WHO. Botulism. WHO. http://www.who.int/mediacentre/factsheets/fs270/en/ [Accessed 25-May-2017]

[22] Surveillance Atlas http://atlas.ecdc.europa.eu/public/index.aspx [Accessed 25-May-2017]

[23] National Botulism Surveillance. Botulism. CDC. https://www.cdc.gov/botulism/surveillance.html. [Accessed 25-May-2017]

[24] Duplantier AJ, Kane CD, Bavari S. Searching for therapeutics against botulinum neurotoxins: A true challenge for drug discovery. Current Topics in Medicinal Chemistry. 2016;**16**:2330-2349

[25] Wein LM, Liu Y. Analyzing a bioterror attack on the food supply: The case of botulinum toxin in milk. Proceedings of the National Academy of Sciences of the United States of America. 2005;**102**:9984-9989

[26] Holtcamp W. One study, two paths: The challenge of dual-use research. Environmental Health Perspectives. 2012;**120**:A238-A242

[27] Enserink M. Biosecurity. As new botulism threat implodes, more questions. Science. 2015;**347**:934-935

[28] Hanchanale VS, Rao AR, Martin FL, et al. The unusual history and the urological applications of botulinum neurotoxin. Urologia Internationalis. 2010;**85**:125-130

[29] Smith TJ, Hill KK, Raphael BH. Historical and current perspectives on *Clostridium botulinum* diversity. Research in Microbiology. 2015;**166**:290-302

[30] Terilli RR, Moura H, Woolfitt AR, et al. A historical and proteomic analysis of botulinum neurotoxin type/G. BMC Microbiology. 2011;**11**:232

[31] Pellett S, Tepp WH, Bradshaw M, et al. Purification and characterization of botulinum neurotoxin FA from a genetically modified *Clostridium botulinum* strain. mSphere. 2016:e00100-15

[32] Smith TJ, Hill KK, Xie G, et al. Genomic sequences of six botulinum neurotoxin-producing strains representing three clostridial species illustrate the mobility and diversity of botulinum neurotoxin genes. Infection, Genetics, and Evolution- Journal of Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases. 2015;**30**:102-113

[33] Giménez DF. Serological identification of botulinum neurotoxins: A critical overview. Anaerobe. 2016;**40**:28-30

[34] Hill KK, Xie G, Foley BT, et al. Genetic diversity within the botulinum neurotoxin-producing bacteria and their neurotoxins. Toxicon Official Journal of International Society on Toxinology. 2015;**107**:2-8

[35] Peck MW, Smith TJ, Anniballi F, et al. Historical perspectives and guidelines for botulinum neurotoxin subtype nomenclature. Toxins. 2017;**9**:38

[36] Maslanka SE, Lúquez C, Dykes JK, et al. A novel botulinum neurotoxin, previously reported as serotype H, has a hybrid-like structure with regions of similarity to the structures of serotypes A and F and is neutralized with serotype A antitoxin. The Journal of Infectious Diseases. 2016;**213**:379-385

[37] Williamson CHD, Sahl JW, Smith TJ, et al. Comparative genomic analyses reveal broad diversity in botulinum-toxin-producing Clostridia. BMC Genomics. 2016;**17**:180

[38] Rummel A. The long journey of botulinum neurotoxins into the synapse. Toxicon Official Journal of International Society on Toxinology. 2015;**107**:9-24

[39] Mazuet C, Legeay C, Sautereau J, et al. Diversity of group I and II *Clostridium botulinum* strains from France including recently identified subtypes. Genome Biology and Evolution. 2016;**8**:1643-1660

[40] Singh BR, Chang T-W, Kukreja R, et al. The botulinum neurotoxin complex and the role of ancillary proteins. In: Foster KA, editors. Molecular Aspects of Botulinum Neurotoxin. New York: Springer Science +Business Media. pp. 69-101

[41] Lam K-H, Jin R. Architecture of the botulinum neurotoxin complex: A molecular machine for protection and delivery. Current Opinion in Structural Biology. 2015;**31**:89-95

[42] Mansfield MJ, Adams JB, Doxey AC. Botulinum neurotoxin homologs in non-Clostridium species. FEBS Letters. 2015;**589**:342-348

[43] Zornetta I, Azarnia Tehran D, Arrigoni G, et al. The first non *Clostridial botulinum*-like toxin cleaves VAMP within the juxtamembrane domain. Scientific Reports. 2016;**6**:30257

[44] Montal M. Botulinum neurotoxin: A marvel of protein design. Annual Review of Biochemistry. 2010;**79**:591-617

[45] Archana MS. Toxin yet not toxic: Botulinum toxin in dentistry. The Saudi Dental Journal. 2016;**28**:63-69

[46] Eisele K-H, Fink K, Vey M, et al. Studies on the dissociation of botulinum neurotoxin type A complexes. Toxicon Official Journal of International Society on Toxinology. 2011;**57**:555-565

[47] Simpson L. The life history of a botulinum toxin molecule. Toxicon Official Journal of International Society on Toxinology. 2013;**68**:40-59

[48] Pirazzini M, Azarnia Tehran D, Leka O, et al. On the translocation of botulinum and tetanus neurotoxins across the membrane of acidic intracellular compartments. Biochimica et Biophysica Acta. 2016;**1858**:467-474

[49] Lam K-H, Yao G, Jin R. Diverse binding modes, same goal: The receptor recognition mechanism of botulinum neurotoxin. Progress in Biophysics and Molecular Biology. 2015;**117**:225-231

[50] Kumaran D, Eswaramoorthy S, Furey W, et al. Domain organization in *Clostridium botulinum* neurotoxin type E is unique: Its implication in faster translocation. Journal of Molecular Biology. 2009;**386**:233-245

[51] Odumosu O, Nicholas D, Yano H, et al. AB toxins: A paradigm switch from deadly to desirable. Toxins. 2010;**2**:1612-1645

[52] Montecucco C. How do tetanus and botulinum toxins bind to neuronal membranes?. Trends in Biochemical Sciences. 1986;**11**:314-317

[53] Hamark C, Berntsson RP-A, Masuyer G, et al. Glycans confer specificity to the recognition of ganglioside receptors by botulinum neurotoxin A. Journal of the American Chemical Society. 2017;**139**:218-230

[54] Kammerer RA, Benoit RM. Botulinum neurotoxins: New questions arising from structural biology. Trends in Biochemical Sciences. 2014;**39**:517-526

[55] Karalewitz AP-A, Fu Z, Baldwin MR, et al. Botulinum neurotoxin serotype C associates with dual ganglioside receptors to facilitate cell entry. The Journal of Biological Chemistry. 2012;**287**:40806-40816

[56] Jacky BPS, Garay PE, Dupuy J, et al. Identification of fibroblast growth factor receptor 3 (FGFR3) as a protein receptor for botulinum neurotoxin serotype A (BoNT/A). PLoS Pathogens. 2013;**9**:e1003369

[57] Mahrhold S, Bergström T, Stern D, et al. Only the complex N559-glycan in the synaptic vesicle glycoprotein 2C mediates high affinity binding to botulinum neurotoxin serotype A1. The Biochemical Journal. 2016;**473**:2645-2654

[58] Fu Z, Chen C, Barbieri JT, et al. Glycosylated SV2 and gangliosides as dual receptors for botulinum neurotoxin serotype F. Biochemistry (Mosc). 2009;**48**:5631-5641

[59] Simpson LL. Kinetic studies on the interaction between botulinum toxin type A and the cholinergic neuromuscular junction. The Journal of Pharmacology and Experimental Therapeutics. 1980;**212**:16-21

[60] Beard M. Translocation, entry into the cell. In: Foster KA, editors. Molecular Aspects of Botulinum Neurotoxin. New York: Springer Science +Business Media; 2014. pp. 151-170

[61] Zanetti G, Azarnia Tehran D, Pirazzini M, et al. Inhibition of botulinum neurotoxins interchain disulfide bond reduction prevents the peripheral neuroparalysis of botulism. Biochemical Pharmacology. 2015;**98**:522-530

[62] Azarnia Tehran D, Pirazzini M, Leka O, et al. Hsp90 is involved in the entry of clostridial neurotoxins into the cytosol of nerve terminals. Cellular Microbiology. 2017;**19**:e12647

[63] Rummel A. Two feet on the membrane: Uptake of clostridial neurotoxins. Current Topics in Microbiology and Immunology. In: Current Topics in Microbiology and Immunology. Berlin: Springer; 2016. pp 1-37

[64] Hong W, Lev S. Tethering the assembly of SNARE complexes. Trends in Cell Biology. 2014;**24**:35-43

[65] Chen S, Barbieri JT. Protease activity of the botulinum neurotoxins. In: Foster KA, editors. Molecular Aspects of Botulinum Neurotoxin. New York: Springer Science +Business Media; 2014. pp. 171-189

[66] Meng J, Wang J, Steinhoff M, et al. TNFα induces co-trafficking of TRPV1/TRPA1 in VAMP1-containing vesicles to the plasmalemma via Munc18-1/syntaxin1/SNAP-25 mediated fusion. Scientific Reports. 2016;**6**:21226

[67] Shoemaker CB, Oyler GA. Persistence of Botulinum neurotoxin inactivation of nerve function. Current Topics in Microbiology and Immunology. 2013;**364**:179-196

[68] Kuo C-L, Oyler GA, Shoemaker CB. Accelerated neuronal cell recovery from botulinum neurotoxin intoxication by targeted ubiquitination. PLoS ONE. 2011;**6**:e20352

[69] Rossetto O, Pirazzini M, Montecucco C. Current gaps in basic science knowledge of botulinum neurotoxin biological actions. Toxicon Official Journal of International Society on Toxinology. 2015;**107**:59-63

[70] Fischer A, Mushrush DJ, Lacy DB, et al. Botulinum neurotoxin devoid of receptor binding domain translocates active protease. PLoS Pathogens. 2008;**4**:e1000245

[71] Pellett S, Tepp WH, Scherf JM, et al. Botulinum neurotoxins can enter cultured neurons independent of synaptic vesicle recycling. PLoS ONE. 2015;**10**:e0133737

[72] Restani L, Giribaldi F, Manich M, et al. Botulinum neurotoxins A and E undergo retrograde axonal transport in primary motor neurons. PLoS Pathogens. 2012;**8**:e1003087

[73] Matak I, Lacković Z. Botulinum toxin A, brain and pain. Progress in Neurobiology. 2014;**119**-**120**:39-59

[74] Matak I, Lacković Z. Botulinum neurotoxin type A: Actions beyond SNAP-25? Toxicology. 2015;**335**:79-84

[75] Pickett A. Botulinum toxin as a clinical product: Manufacture and pharmacology. In: Foster KA, editors. Clinical Applications of Botulinum Neurotoxin. New York: Springer Science +Business Media; 2014. pp. 7-49

[76] Weisemann J, Krez N, Fiebig U, et al. Generation and characterization of six recombinant botulinum neurotoxins as reference material to serve in an International proficiency test. Toxins. 2015;**7**:5035-5054

[77] Naumann M, Boo LM, Ackerman AH, et al. Immunogenicity of botulinum toxins. Journal of Neural Transmission. 2013;**120**:275-290

[78] Fabbri M, Leodori G, Fernandes RM, et al. Neutralizing antibody and botulinum toxin therapy: A systematic review and meta-analysis. Neurotoxicity Research. 2016;**29**:105-117

[79] Yablon SA, Brashear A, Gordon MF, et al. Formation of neutralizing antibodies in patients receiving botulinum toxin type a for treatment of poststroke spasticity: A pooled-data analysis of three clinical trials. Clinical Therapeutics. 2007;**29**:683-690

[80] Kane CD, Nuss JE, Bavari S. Novel therapeutic uses and formulations of botulinum neurotoxins: A patent review (2012-2014). Expert Opinion on Therapeutic Patents. 2015;**25**:675-690

[81] Eleopra R, Tugnoli V, Quatrale R, et al. Clinical use of non-A botulinum toxins: botulinum toxin type C and botulinum toxin type F. Neurotoxicity Research. 2006;**9**:127-131

[82] Cossins A, Beard M. Recombinant Clostridium Botulinum Neurotoxins. WO/2014/068317 https://patentscope.wipo.int/search/en/detail.jsf?docId= WO2014068317. 2014 [Accessed 25-May-2017]

[83] Dong M, Peng L, Stenmark PEG, et al. Engineered Botulinum Neurotoxin. WO/2013/180799https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2013180799&recNum=112&docAn=US2013030737&queryString=CALCITONIN*%20OR%20PTH%20OR%20PTHRP%20OR%20PARATHYROID*%20OR%20FASUDIL*%20OR%20NAFTOPIDIL*%20OR%20MILNACUPRAN*%20OR%20BREDININ*%20OR%20MIZORIBIN*%20OR%20FAMCILOVIR*%20OR%20THROMBOMODULIN*&maxRec=21893. 2013 [Accessed 25-May-2017]

[84] Johnson E, Tepp W, Lin G. Purification, Characterization, and Use of Clostridium Botulinum Neurotoxin Bont/A3. WO/2013/049139 https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2013049139&recNum=73&docAn=US2012057243&queryString=&maxRec=129958. 2013 [Accessed 25-May-2017]

[85] Kaji R. Clinical differences between A1 and A2 botulinum toxin subtypes. Toxicon Official Journla of International Society on Toxinology. 2015;**107**:85-88

[86] Mukai Y, Shimatani Y, Sako W, et al. Comparison between botulinum neurotoxin type A2 and type A1 by electrophysiological study in healthy individuals. Toxicon Official Journla of International Society on Toxinology. 2014;**81**:32-36

[87] Foran PG, Mohammed N, Lisk GO, et al. Evaluation of the therapeutic usefulness of botulinum neurotoxin B, C1, E, and F compared with the long lasting type A. Basis for distinct durations of inhibition of exocytosis in central neurons. The Journal of Biological Chemistry. 2003;**278**:1363-1371

[88] Foster K, Chaddock J, Marks P, et al. Fusion Proteins. WO/2006/059093 https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2006059093. 2006 [Accessed 23-January-2017]

[89] Foster K, Chaddock J. Targeted secretion inhibitors—Innovative protein therapeutics. Toxins. 2010;**2**:2795-2815

[90] Sutton JM, Wayne J, Scott-Tucker A, et al. Preparation of specifically activatable endopeptidase derivatives of *Clostridium botulinum* toxins type A, B, and C and their applications. Protein Expression and Purification. 2005;**40**:31-41

[91] Masuyer G, Beard M, Cadd VA, et al. Structure and activity of a functional derivative of *Clostridium botulinum* neurotoxin B. Journal of Structural Biology. 2011;**174**:52-57

[92] Chaddock JA, Purkiss JR, Friis LM, et al. Inhibition of vesicular secretion in both neuronal and nonneuronal cells by a retargeted endopeptidase derivative of *Clostridium botulinum* neurotoxin type A. Infection and Immunity. 2000;**68**:2587-2593

[93] Foster KA, Adams EJ, Durose L, et al. Re-engineering the target specificity of clostridial neurotoxins–A route to novel therapeutics. Neurotoxicity Research. 2006;**9**:101-107

[94] Somm E, Bonnet N, Martinez A, et al. A botulinum toxin-derived targeted secretion inhibitor downregulates the GH/IGF1 axis. The Journal of Clinical Investigation. 2012;**122**:3295-3306

[95] Lee S-M, Booe JM, Pioszak AA. Structural insights into ligand recognition and selectivity for class A, B, and C GPCRs. European Journal of Pharmacology. 2015;**763**:196-205

[96] Somm E, Bonnet N, Zizzari P, et al. Comparative inhibition of the GH/IGF-I axis obtained with either the targeted secretion inhibitor SXN101959 or the somatostatin analog octreotide in growing male rats. Endocrinology. 2013;**154**:4237-4248

[97] Leggett J, Harper E, Waite E, et al. GHRH receptor-targeted botulinum neurotoxin selectively inhibits pulsatile GH secretion in male rats. Endocrinology. 2013;**154**:3305-3318

[98] Fonfria E, Donald S, Cadd VA. Botulinum neurotoxin A and an engineered derivate targeted secretion inhibitor (TSI) A enter cells via different vesicular compartments. Journal of Receptor and Signal Transduction Research. 2016;**36**:79-88

[99] Home - ClinicalTrials.govhttps://clinicaltrials.gov/ [Accessed 5-January-2017]

Chapter 6

Resistance to Botulinum Toxin in Aesthetics

Sebastián Torres Farr

Additional information is available at the end of the chapter

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Abstract

Botulinum toxins are widely used worldwide for the treatment of medical and esthetic disorders. They are considered the gold standard for the treatment of muscular spasticity and facial dynamic wrinkles. Moreover, they are a valid alternative in the treatment of pain and hyperhidrosis. Several adverse events to their applications have been described, being the most frequent hematomas, migraines, palpebral ptosis, ectropion, or lack of response. Resistance to botulinum toxins in the medical field has been described, at higher dosages and short intervals. Nevertheless, resistance to botulinum toxin in esthetics has been considered traditionally anecdotic. Recent evidence suggests that resistance to botulinum toxins in esthetics may have higher prevalence than expected. A full analysis of the argument is given with up dated information regarding botulinum toxin resistance in medical and esthetic arenas; including elements for suspicion and diagnosis, valid alternatives for effective prevention, education and treatment of this misdiagnosed condition.

Keywords: resistance, neutralizing antibodies, botulinum toxin, proteins

1. Introduction

When botulinum toxin (BTX) was first introduced to esthetics, it was after an observation related to its side effects in the context of blepharospasms treatments [1]. This fact, as with many other drugs permitted the study, development and use of this tool into another area of medicine, such as cosmetic surgery.

The exponential growth of the technique, due to the medical community and patient's interests, increased the knowledge surrounding this molecule, and the safety concerns that called attention at the beginning of the century due to the use of a toxin in esthetics. Moreover, it permitted the study of the adverse events related to the applications [2, 3]. Among the former the most common include; hematomas, migraines, palpebral ptosis, ectropion, or lack of response. A complete list is shown in **Table 1**.

Any physician with some experience in the esthetic field understands the importance of patient satisfaction. Whenever we get a patient complaint after a BTX treatment, such as in cases of none or partial response, we face an immediate judge regarding trust, loyalty and professionalism. For these reasons, it is of prime importance to educate or patients concerning the possible causes of their complaint.

The causes of lack of response include: drug potency, toxin type, injection technique, unexpressive patients, cold chain alteration, toxin condition, dilution, insufficient dose, unreal expectations and resistance [4]. The causes are summarized in **Table 2**.

With the introduction to the market to new BTX type A, we have found empirically, that even though each toxin claims to have unique and non-interchangeable units, there are toxins that are more powerful than others in clinical response and lasting effect. This has called the attention to the **drug potency** parameter, especially when patients are switched from toxins between different treatments [5].

Clostridium botulinum bacteria produces seven serologically distinct types of botulinum neurotoxin (designated as types A, B, C1, D, E, F, and G). All subtypes of toxin act by preventing the release of acetylcholine at the neuromuscular junction. The most potent and used **toxin type** is A. It is clear that when changing the type, very rare condition, we can expect a lesser response to the latter.

BT treatments adverse events
Pain
Hematoma, bruising, inflammation
Headaches
Palpebral ptosis
Pseudo ptosis
Drug interaction
Diplopia
Asymmetries
Ectropion
Allergies
Unsatisfied patient
Muscular atrophy
Dysphagia
Resistance

Table 1. Botulinum toxin (BT) treatment adverse events.

BT lack of response
Drug potency
Toxin type
Injection technique
Unexpressive patients
Cold chain
Toxin condition
Dilution
Insufficient dose
Un real expectations
Resistance

Table 2. Possible causes of lack of action of botulinum toxin (BT).

Technical errors during the procedure such as intra dermal toxin injections or placement distant to the target muscle can manifest as lack or partial response in affected areas. Adequate touch ups should guarantee patient overall satisfaction.

Unexpressive patients are not good candidates for BTX treatments, because they seldom have problems with dynamic wrinkles, and therefore are difficult in perceiving post procedure improvements with the technique.

The **cold chain** can be a major concern for BTX that are thermo-sensitive. Viability of this type of BTX at room temperature is around 240 h. According to Allegan, during BTX production, a total of 120 h are consumed at room temperature for production, while the remaining 120 h enables logistics (shipping and delivery to destination). Whenever the cold chain is not respected, it is highly probable that part of the vial content is damaged, therefore affecting the performance of it.

To guarantee indemnity storage should be done frozen (−5 to 8°C) until used and after reconstitution refrigerated at 2–8°C. The use of non-thermo-sensitive BTX could be advisable if Cold chain rupture is an issue.

Toxin condition can be altered whenever one of the following is verified: reconstitution >6w (according to Dr. Doris Hexsel's publications), alcohol contact with toxin during disinfection, application of ice packs after treatment (favors vasoconstriction and lesser absorption), freezing the toxin after reconstitution (thermic shock damages toxin), or energic shake of the vial after reconstitution. Gentle handling and attention to this details should allow to prevent this event.

Dilution has been traditionally considered as a personal preference of the physician. Nevertheless, recent communications by this author have questioned this and open debate regarding the possibility of augmenting the potency of the drug by reducing the amount of solvent in the reconstitution, or better said, by concentrating the toxin. The effects obtained

through this are lesser side effects (such as migration) and prompt, more important and long lasting clinical benefits. The author recommends 0.5 ml as reconstitution for 125 Speywood Units (Azzalure-Galderma) or 50 Botox units (Vistabex-Allergan) or 1 ml for 100 Botox U (Botox-Allergan) or 2 ml for 300 Speywood Units (Dysport-Ipsen).

Doses are gender and individual specific. Most of the textbooks available regarding esthetic applications of BTX present recipes or treatment forms with practical advices about dose and target muscles. Nevertheless, all of this represent general recommendations to orient the clinician to target the specific dose for the specific patient. In this way, a correct clinical response is obtained without over or under treatment of the areas involved.

Patient with **unreal expectations** are poor candidates for BTX treatments. A correct clinical history, physical exam and discussion of the procedure with photographs, of previous treatments, will help to rule out them and avoid complications.

2. Resistance to chemodenervation definition

This condition is defined as the absence of benefic response and muscular atrophy after BTX treatment, due to an antibody response (of neutralizing antibodies) to the protein content within the product that neutralizes the effect.

The antibodies anti-BTX can be:

i. Neutralizing

ii. Non-neutralizing: they do not influence directly on the therapeutic effect, but augment foreign protein charge, increasing potentially the formation of neutralizing antibodies.

All above rank the protein content within a BTX vial as a potential trigger to develop resistance. Although there are areas within the progenitor toxin complex (PTC) that have been identified as more prone to develop neutralizing antibodies, potentially all proteins could develop an immune response that may interfere action and develop resistance. The proteins within a vial are classified in two groups:

- Structural proteins: they are present within the toxin complex and include peripheral proteins (hemagglutinin and nonhemagglutinin proteins) and core proteins of the neurotoxin itself. The diagram of the toxin is shown in **Figure 1**.
- The role of these peripheral proteins also known as Nontoxic neurotoxin associated proteins (NAPS) is related to the oral ingestion of the toxin. Non-toxic-nonhemagglutinin proteins (NTNHA) protect the progenitor toxin complex against digestive proteases and gastro intestinal acidic ambient, whereas hemagglutinin proteins (HA) enhance intestinal absorption of it [6].

The neurotoxin molecule is a 150 kDa protein structure made up of a 100 kDa heavy chain and 50 kDa light chain held together by a disulfide bond and associated with a zinc atom. The heavy chain has the C-terminus on it and is responsible for the high affinity docking on the presynaptic nerve membrane, being it rapid and irreversible. The light chain is responsible

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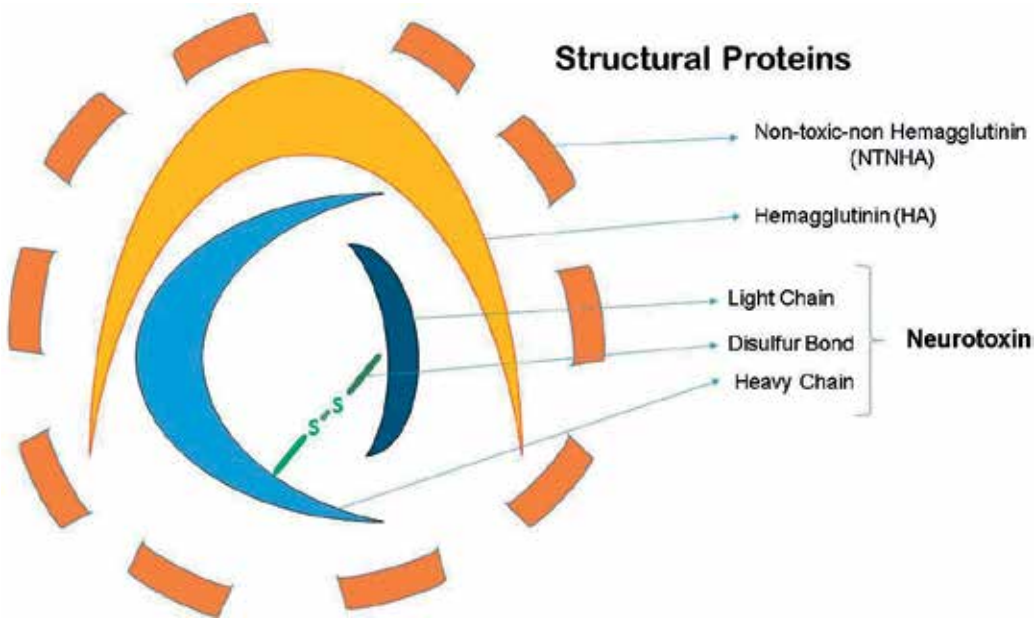

Figure 1. Diagram of progenitor toxin complex evidencing structural proteins (NTNHA, HA and neurotoxin).

Product	Toxin type	Lab	Formulation	Units/vial	Excipients	Storage
Azzalure	A	Galderma	Lyophilisate	125 Speywood	125 μg SA 2.5 mg Lactose	2–8°C
Dysport	A	Ipsen	Lyophilisate	300 Speywood	125 μg SA 2.5 mg Lactose	2–8°C
Vistabex	A	Allergan	Vaccum dried powder	50 Allergan	500 μg SA 0.9 mg NaCl	2–8°C
Botox	A	Allergan	Vaccum dried powder	100 Allergan	500 μg SA 0.9 mg NaCl	2–8°C
Bocouture	A	Merz	Lyophilisate	50 Merz	1000 μg SA Cane sugar 5 mg	RT
Xeomin	A	Merz	Lyophilisate	100 Merz	1000 μg SA 25 mg Sucrose	RT
CBTX-A*	A	Lanzho Institute of Biological Products	Lyophilisate	100	20 mg gelatin 25 mg dextran 25 mg SUCROSE	−5/−20°C
Myobloc/ Neurobloc	B	Solstice	Liquid	5000, 10,000, or 20,000	500 μg/ml SA 10 nM Na succinate 100 nM Na chloride, Na octanoate, pH = 5.6	2–8°C

*CBTX-A, Chinese BoNT/A complex; SA, serum albumin, RT, room temperature.

Table 3. Principal characteristics of most common BTX products available.

for the intracellular cleavage of proteins required for transmission of acetylcholine across the neuromuscular junction. Regions of the light chain have been recognized by human antitoxin antibodies from cervical dystonia patient's immunoresistant to toxin treatment [7–11]. The antigenic structure of the active toxin recognized by human antibodies relies predominantly on three peptides within the light chain named L11, L14 and L18. These three antigenic regions reside in close proximity to the belt of the heavy chain. The regions L11 and L18 are accessible in both the free light chain and the holotoxin forms, while L14 appears to be less accessible in the holotoxin. Antibodies against these regions could prevent delivery of the L-chain into the neurons by inhibition of the translocation.

- Excipients proteins: they are present in most commercial products available in the market in the form of serum albumin, the only exception being the Chinese BTX-A, that uses gelatin as protein stabilizer. This plasmatic transport protein is regularly used in hemodynamic treatments as a volume stabilizer. Moreover, it is present in diary and eggs and is susceptible of allergenic response. Nevertheless, most of the allergic reactions or intolerances are due to lacto globulin and not serum albumin. The role of this protein in the vials serves especially as protein stabilizer to prevent the adsorption of the toxin to glass and plastic surfaces and probably to maintain the toxin in the target area after being injected. In this case, according to the author theory, would be the principal responsible of limiting micro diffusion. On the other hand, macro diffusion, associated with the most feared complication such as palpebral ptosis, depends on the solvent quantity (or dilution) for the vial preparation [12].

The other excipients are not important regarding resistance to BTX. Details of the principal characteristics of the most common BTX products available in the market is given in **Table 3**. Excipients within the vials respect a very strict proportion, and when altered could affect lasting effect.

3. BTX resistance risk factors

The principal aspects recognized are:

- Higher doses: this characteristic exposes medical patients to a major risk in comparison to esthetic applications, and wrongly induced physicians to believe that resistance was not possible in the esthetic field [13–15]. As recently demonstrated in the author's publication, resistance in esthetics is not only possible, but more frequent than expected.

- Short treatment intervals: according to this feature, treatments should only be done every 6 months if possible and touch ups should be limited within the first week and only once.

- Individual predisposition: some patients are more susceptible than others in resistance development. As with many autoimmune disorders, females seem more prone to be affected, although males usually are treated with higher doses of BTX. A correct clinical history may identify allergic or autoimmune disorders, which could put the patient at increased risk of

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resistance and could constitute a relative contraindication for the treatment. The exact phenotype of this patients have not been fully understood or identified yet, and genetic tests could give some information about it in the future.

4. Diagnosis

BTX resistance is based in 2 pillars: clinical suspicion and laboratory tests.

The former is of vital importance as you can only diagnose what you have been trained to see or suspect. Patients present generally upset, after 2 or more BTX treatments in which they refer that the treatment did not gave the expected result, did not work or the intensity or duration of the clinical response was less than expected or experimented in previous treatments.

The common physician action here is to raise the dose previously used or to change the commercial preparation employed (always staying in BTX-A), increasing both patient anxiety and resistance.

Laboratory test can be clinical, in vivo or in vitro [16].

Clinical tests are intramuscular measurements of compound potential action and are indirect and not specific.

In vivo tests are considered the gold standard for the diagnosis, since they represent the only FDA approved. The most popular is the bioassay or mouse lethality test. They give quantitative results about the presence of Antibodies in the patient's serum. The test consist in the injection of the suspected patient serum intravenously or intra peritoneal in mouse together with the inoculation of BTX-A. Toxicity or death of the animals is measured and is expected if no or low titers antibodies are present within the patient serum.

It has medium sensibility and specificity, takes 2–4 days, and is rather expensive (140 Euros). Moreover, is complicated (not available in many regions), and has obvious animal rights issues.

The test is available in Hannover Germany in Toxogen Lab (www.toxogen.de). The sample of patient blood (14 ml) or serum (5 ml) not frozen nor heparinized should be sent in a cold plastic container through express mail. The test form is presented in **Figure 2**.

In vitro tests include the mouse diaphragm assay (MDA), enzyme-linked immunosorbent assays (ELISA) or the immunoprecipitation assay (IPA). They are not specific and measure only one part of the reaction.

A new in vitro test known as Neuronal Cell-based Botulinum Neurotoxin Assay, based on histological preparations of mouse neural cord is available. It guarantees less animal's death and sufferance (72 tests/animal) and is highly sensitive, specific and safe. For these reasons, it may be the test of the future [17].

A summary of diagnostic tests is given in **Table 4**.

toxogen Diagnostik GmbH

toxogen GmbH
Feodor-Lynen-Straße 35
30625 Hannover

Order Form

Please test submitted sample(s) for antibodies directed against Botulinum toxin

Type A ☐
Type B ☐ please mark box with a cross where applicable

Patient's particulars

Name:	
First name:	Date of birth:

Treatment

Indication:	
Dose:	Total dose:
Time between injections:	Duration of treatment:

Sender

Doctor:	Phone:
E-Mail:	Fax:
Hospitel:	Ward:
Address:	Postal code:
	Country:

Recipient of invoice (if not identical with sender)

Name:	First name:
Address:	Postal code:
	Country:

Date: _____ Signature: _____

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Amtsgericht Hannover
HRB-Nr. 56383
USt-ID Nr. DE 189154617

Geschäftsführer:
Prof. Dr. H. Bigalke
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Figure 2. Resistance test form.

Type	Name	Characteristics
Clinical	Compound Action Potential	Intramuscular measurements (Frontalis, Extensor Digitorum Brevis, SCM) Indirect ↓Sp
In vivo	Mouse Lethality Test	Gold Std, quantitative, FDA approved Medium Se/Sp, complicated Expensive 2–4 days Animal rights
In vitro	MDA ELISA IPA	↓Sp Single phase measurements
	Neuronal Cell-based Botulinum Neurotoxin Assay	Histologic sample of rat neural cord <N° (72 test/animal) & animal suffering ↑Se/Sp Safer, future

SCM, sternocleidomastoid muscle; ↑, high; ↓, low; Se, sensitivity; Sp, specificity; MDA, mouse diaphragm assay; ELISA, enzyme-linked immunosorbent assays; IPA, immunoprecipitation assay.

Table 4. Diagnostic test for BTX-A resistance.

5. Prevention

The prophylaxis for resistance development includes: adequate treatment intervals, personalized treatments, minimal effective dose, periodical tests execution, patient education, vast therapeutic arsenal, treatment onset >30 years (esthetic) and to avoid patients with strong allergic or autoimmune phenotype.

As previously mention, treatment intervals should be 6 months, with the minimum possible interval around 3 months, and touch should be done within the first week after the treatment and only once.

The treatments should be personalized, escaping from traditional formulas, using the minimal effective dose for the target area and avoiding treating areas with little or no mobility or wrinkles (example: procerus muscle in some patients). It is important to keep track on the individual treatment schema and lasting of the clinical effect, as the duration can be the first indicator that there is some resistance development.

In case of suspicion, serologic test should be promptly indicated.

Patients should be informed about this condition and about the other therapeutic alternatives, like bioplasties or laser, which can be combined to avoid the neurotoxin abuse, or "neurotoxin dependent syndrome". In this way we will obtain natural results and maintain facial mobility without wrinkles. The idea is to make patients to consider both; skin quality and facial wrinkles.

Some congress communications in Latin America, have shown the use of BTX in 16 years old patients for wrinkles prevention. In this case, although it may be effective, it would expose the patient to a very long way on toxin treatments, and potential resistance [18]. For this reason,

in this group wrinkles prevention is advised through a correct cosmetic skin care, sun protection and nutrition. We recommend cosmetic BTX treatments not to be started before the 3rd decade of life.

Finally, within the clinical history attention should focus on the autoimmune and allergic phenotypes. Although it is not quite clear, both groups would have an important tendency to develop resistance and should be considered a partial contraindication for BTX applications.

Each case should be discussed and cost benefit decision should be taken with an informed patient.

6. Treatment

Treatment alternatives include botulinum toxin type B (BTX-B), medical electronic and infiltrative devices, peelings and cosmetic skin care. A special mention should be given to psychological support for the patient with BTX resistance.

BTX-B, also known as Myobloc (US) or Neurobloc (EC) (Solstice Laboratories) is a toxin of diverse type and although it has a quicker onset than BTX-A is not as potent and the effects will last less. Even though antibody titers tend to diminish in time, new treatments with BTX-A are not suggested. Other important fact to mention is that the drug is intended in Europe for Cervical Dystonia, and not for esthetic use, so it is very difficult to obtain the product in a private practice setting.

Medical electronic devices embrace non-ablative or ablative procedures. Non-ablative methodologies include infrared, radiofrequency and localized ultrasounds, only to name a few. Ablative procedures include lasers, like CO_2 or erbium.

All above improve collagen synthesis and skin reorganization and may increase dermic thickness reducing visible wrinkles.

Infiltrative devices group bioplasties (to improve skin hydration or metabolism), or needling through derma rollers or dermic pens.

Peelings may enhance skin renewal and tone. They may be superficial, intermediate or deep.

Cosmetic skin care should always be present in our esthetic practice; it will enhance and maintain our results and is considered to be the single most important measure in skin antiaging. The regimen should consider adequate detergent, day and night time topical preparations and should be adjusted according to the skin type and patients' habits.

Finally, it is important to consider the physiological implications of the patient BTX resistant. For many of them, it is considered a major loss, especially if they were used to regular treatments and had experimented its benefits for a long time. It is suggested to listen to the patient fears and to provide alternative options, to avoid helpless or abandon feelings. Psychological support with specialist is advised if during controls, patients is perceived extremely anxious, hesitated or depressed.

7. Conclusion

Botulinum toxin is one of the best therapeutic weapons available for the treatment of facial dynamic wrinkles and we must, if possible, prevent, suspect, diagnose and treat the presence of resistance, to perpetuate a safe and efficient use of the product for our patients in the future.

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References

[1] Gil Polo C, Rodríguez Sanz MF, Berrocal Izquierdo N, et al. Blepharospasm and hemifacial spasm: Long-term treatment with botulinum toxin. Neurología. 2013 Apr;**28**(3):131-136

[2] Chen S. Clinical uses of botulinum neurotoxins: Current indications, limitations and future developments. Toxins (Basel). 2012 Oct;**4**(10):913-939

[3] Lowe NJ. Overview of botulinum neurotoxins. Journal of Cosmetic and Laser Therapy. 2007;**9**(Suppl 1):11-16

[4] Torres S, Hamilton M, Sanches E, et al. Neutralizing antibodies to botulinum neurotoxin type A in aesthetic medicine: Five case reports. Clinical, Cosmetic and Investigational Dermatology. 2013 Dec 18;**7**:11-17

[5] Pérez-Saldaña MT, Tan EK, Burguera JA. Long-term treatment outcome with different formulations of botulinum toxin. European Journal of Neurology. 2009 Mar;**16**(3):285-286

[6] Yao G, Lee K, Gu S, et al. Botulinum neurotoxin A complex recognizes host carbohydrates through its hemagglutinin component. Toxins (Basel). 2014 Feb 12;**6**(2):624-635

[7] Atassi MZ, Dolimbek BZ, Jankovic J, et al. Regions of botulinum neurotoxin A light chain recognized by human anti-toxin antibodies from cervical dystonia patients immunoresistant to toxin treatment. The antigenic structure of the active toxin recognized by human antibodies. Immunobiology. 2011 Jul;**216**(7):782-792

[8] Atassi MZ, Dolimbek BZ, Jankovic J, et al. Molecular recognition of botulinum neurotoxin B heavy chain by human antibodies from cervical dystonia patients that develop immunoresistance to toxin treatment. Molecular Immunology. 2008 Sep;**45**(15):3878-3888

[9] Lungu C, Karp BI, Alter K, et al. Long-term follow-up of botulinum toxin therapy for focal hand dystonia: Outcome at 10 years or more. Movement Disorders. 2011 Mar;**26**(4):750-753

[10] Ruiz PJ, Castrillo JC, Burguera JA, et al. Evolution of dose and response to botulinum toxin A in cervical dystonia: A multicenter study. Journal of Neurology. 2011 Jun;**258**(6):1055-1057

[11] Brin MF, Comella CL, Jankovic J, et al. Long-term treatment with botulinum toxin type A in cervical dystonia has low immunogenicity by mouse protection assay. Movement Disorders. 2008 Jul 30;**23**(10):1353-1360

[12] Reynolds C. Botulinum Toxin and Botulism. 1st ed. Nova Biomedical. NY, USA. 2015; 41 p. ISBN: 9781634833844

[13] Pistolesi D, Giannarini G, Stampacchia G, et al. Should we stop using botulinum neurotoxin type A in patients with neurogenic detrusor overactivity after development of secondary resistance? European Urology. 2011 Oct;**60**(4):872-873

[14] Dowson C, Khan MS, Dasgupta P, et al. Repeat botulinum toxin-A injections for treatment of adult detrusor overactivity. Nature Reviews. Urology. 2010 Dec;**7**(12):661-667

[15] Kajbafzadeh AM, Nikfarjam L, Mahboubi AH, et al. Antibody formation following botulinum toxin type A (Dysport) injection in children with intractable bladder hyperreflexia. Urology. 2010 Jul;**76**(1):233-237

[16] Bachhuber A, Wagner A, Reichel G, et al. Proof of botulinum toxin antibodies with the Extensor Digitorum Brevis Test. Der Nervenarzt. 2008 Aug;**79**(8):908-911

[17] Pellett S, Tepp WH, Clancy CM, et al. A neuronal cell-based botulinum neurotoxin assay for highly sensitive and specific detection of neutralizing serum antibodies. FEBS Letters. 2007 Oct 16;**581**(25):4803-4808

[18] Aun MV, Chung TM, Santos KS, et al. Is age associated with the development of antibodies against botulinum toxin? Allergologia et Immunopathologia (Madrid). 2013 Jul-Aug;**41**(4):276-279

Chapter 7

Developmental Neurotoxicity of Fluoride: A Quantitative Risk Analysis Toward Establishing a Safe Dose for Children

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Additional information is available at the end of the chapter

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Abstract

A meta-analysis showed that children with higher fluoride exposure have lower IQs than similar children with lower exposures. Circulating levels of fluoride in blood and urine in children have also been linked quantitatively to significantly lower IQ. Other human and animal studies indicate that fluoride is a developmental neurotoxicant and that it operates *in utero.* Economic impacts of IQ loss have been quantified. The objective was to use data from the meta-analysis and other studies to estimate a daily dose of fluoride that would protect all children from lowered IQ, and to estimate economic impacts. We used two methods: traditional lowest-observed-adverse-effect (LOAEL)/no-observed-adverse-effect level (NOAEL); and benchmark dose (BMD). We used 3 mg/L in drinking water as an "adverse effect concentration," with reported fluoride intakes from food, in the LOAEL/NOAEL method. We used the available dose–response data for the BMD analysis. Arsenic, iodine, and lead levels were controlled for in studies we used. BMD analysis shows the possible safe dose to protect against a five-point IQ loss is between 0.0014 and 0.050 mg/day. The LOAEL/NOAEL safe dose range estimate is 0.0042–0.16 mg/day. The economic impact for IQ loss among US children is loss of tens of billions of dollars.

Keywords: fluoride, developmental neurotoxicity, reference dose, economic impact, risk analysis

1. Introduction

This chapter reports on the work we did in translating extant information on the developmental neurotoxicity of fluoride into a range of reference doses, which are doses that may,

within an order of magnitude, be experienced by children throughout their lifetimes without adverse effect on their neural development. This work has, in slightly different format, form and content been published in the journal *Fluoride*, Vol. 49(4 Pt 1):379–400, December 2016.

Interest in the developmental neurotoxicity of fluoride has grown significantly since the 2006 report of the National Research Council Committee (NRC) on Fluoride in Drinking Water [1] that recommended the U.S. Environmental Protection Agency (USEPA) set a new drinking water standard.

A large body of evidence—over 300 animal and human studies—indicates that the fluoride ion is neurotoxic. This includes over 40 studies published in China, Iran, India, and Mexico [2] that found an association between lowered intelligence quotient (IQ) and exposure to fluoride [3]. A meta-analysis by Choi et al. [4] found that in 26 out of 27 studies, children in the high-exposed community had a lowered mean IQ compared to children in a low-exposed community. However, a recent study, by Broadbent et al. [5] did not find a difference in IQ between children living in an artificially fluoridated community or a non-fluoridated community in New Zealand. In this chapter, we explain the substantial limitations of this latter paper.

We used data from Choi et al. [4] and a set of the best IQ studies from China by Xiang et al. [6–10], which accounted for many important confounding variables, to estimate a reference dose for fluoride using two standard risk analysis techniques used by the USEPA, to protect all children in the USA from lowered IQ. Based on our calculations, the protective daily dose should be no higher than 0.05 mg/day for children aged 8–13. We based our risk analysis primarily on information from China, because scientists in that nation have been by far the most active in generating information on fluoride and children's IQ. We are unaware of any similar studies having been done in the USA.

The study by Broadbent et al. [5] found no statistically significant difference in intelligence between groups of children in fluoridated or non-fluoridated communities in New Zealand. A key limitation of this study is that the difference in fluoride intake between the fluoridated and non-fluoridated communities was small, thereby diminishing the power of the study to detect an effect of fluoride on IQ. Menkes et al. [11] addressed this issue and others in a comprehensive commentary on Broadbent et al. [5]. They concluded that the study, "…appears to have overstated available evidence."

Prominent examples of the growing body of literature indicating that fluoride is a developmental neurotoxicant in humans include studies by Malin and Till [12], Wang et al. [13], Zhang et al. [14], the meta-analysis by Choi et al. [4], and the set of studies by Xiang et al. [6–10].

Malin and Till [12] reported an association between prevalence of artificial water fluoridation and prevalence of attention deficit-hyperactivity disorder (ADHD) in the USA. They determined ADHD and water fluoridation prevalence, state by state, from children's health surveys conducted by the Centers for Disease Control (CDC) and water fluoridation data also from CDC sources. They showed that, after correcting for household income, the incidence of ADHD in the years 2003, 2007, and 2011, measured at the state level, increased as the percentage of each state's population drinking fluoridated water increased, as measured in 1992.

The authors discussed their statistical analytical methods that were able to predict that a 1% increase of water fluoridation incidence over that of 1992 was associated with about 67,000 extra diagnoses of ADHD in 2003, about 97,000 extra diagnoses in 2007, and about 131,000 in 2011. They discussed the limitations of their work, and offered plausible mechanisms by which artificial water fluoridation might cause or contribute to ADHD.

Peckham et al. [15] found depressed thyroid function in areas of England as a function of fluoride levels in drinking water, offering a possible secondary mechanism by which fluoride levels may affect neurological development. They found odds ratios of 1.37 and 1.62 for hypothyroidism in areas where water fluoride levels were >0.3 to ≤0.7 and >0.7 mg/L, respectively. It has been reported that the severity of maternal hypothyroidism is inversely correlated with the IQ of the offspring (Klein et al. [16] in NRC [1]). When iodine intakes are deficient, doses of fluoride of 0.01–0.03 mg/kg/day (equivalent to 0.5–1.5 mg/day for a 50 kg woman) altered thyroid-stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) hormone levels ([1], pp. 262–263). This further indicates that those with iodine deficiency are a sensitive subgroup that USEPA must consider, given the fluoride exposures from all sources for women 13–49 years of age, with drinking water at 1 mg/L of 0.033–0.042 mg/kg/day ([1], Table 2-12.) According to the World Health Organization, WHO [17, 18] median urinary iodine levels <150 μg/L are considered insufficient, and Caldwell et al. [19] reported that 56.9% of pregnant women surveyed in the USA during 2005–2008 had a median urinary iodine concentration < 150 μg/L.

The Zhang et al. study [14] also found a statistically significant elevation of TSH among the children exposed to the "high" water fluoride level (mean 1.40 mg/L; range 1.23–1.58 mg/L) compared to controls (mean 0.63 mg/L; range 0.58–0.68 mg/L).

Wang et al. [13] showed a statistically significant negative relationship between urinary fluoride levels and IQ among children. They examined both fluoride and arsenic as covariates, and showed through determination of urinary fluoride and arsenic levels that fluoride was most likely the source of the effect. They reported a statistically significant IQ difference of 4.3 IQ points between high (n = 106, 5.1 ± 2.0 mg/L) and control (n = 110, 1.5 ± 1.6 mg/L) urinary fluoride groups.

Zhang et al. [14] found a significant negative relationship between both urinary and serum fluoride levels and IQ in children. Further, they showed that a subset of the study cohort with the val/val(158) allele of the catechol-O-methyltransferase (COMT) gene was more susceptible to fluoride reduction of IQ than were the rest of the cohort, who had the two alternate genotype alleles (met/met and val/met) of that gene. This gene codes for the major enzyme involved in the metabolic degradation of dopamine, which is recognized as having an important role in cognition. The two median and inter-quartile ranges of fluoride levels in drinking water were: high 1.46 (range 1.23–1.57); and control 0.60 (range 0.58–0.68) mg/L. Differences between high exposure and control exposure groups for water fluoride, serum fluoride, and urine fluoride level were statistically significant. Both serum fluoride and urine fluoride were significantly related to water fluoride levels, and both were also significantly related to lower IQ. For urinary fluoride levels the IQ point difference from controls was: 2.42 per mg/L (95% C.I. −4.59 to 0.24, $p < 0.05$).

The Choi et al. study [4] identified 39 studies that investigated fluoride exposure levels and neurodevelopmental outcomes in children. Only 27 of these met selection criteria for their meta-analysis. Choi et al. concluded that, "Children who lived in areas with high-fluoride exposure had lower IQ scores than those who lived in low-exposure or control areas," and presented reasons why the conclusion is valid: remarkable consistency; relatively large effect; studies were independent of each other by different researchers and in widely differing areas; and although confounders such as co-exposures to iodine, lead, and arsenic were not considered in some of the studies, they were considered in others. Ten studies from Ref. [4] had mean high-fluoride drinking water levels of less than 3 mg/L, which is lower than the current health-based drinking water standard in the USA [20], discussed below. The average IQ loss among these eight studies was 7.4 points. As described below, the quality of the Choi study and its findings prompted us to examine ways to use and build on it and the Xiang et al. series to try estimating where a safe dose, if any, lay.

One of the studies included in Ref. [4] meta-analysis was by Xiang et al. [6]. The Xiang research group, alone among those cited by Choi et al. [4], published a set of studies referred to above, from which total fluoride doses could be estimated, permitting a dose-response analysis. This was the key to being able to use the benchmark dose method in our analysis, described below, while recognizing the limitations imposed by the relatively small number of children studied. This set of studies by Xiang et al. also included data on co-exposures to lead, arsenic, and iodine, [6, 8, 9] respectively, as well as other potential confounding factors which were accounted for, and we used this set in our work for these reasons.

The studies by Xiang et al. were conducted on 512 children in high-fluoride Wamiao village (n = 222) and low-fluoride Xinhuai village (n = 290). The studies investigated fluoride exposures, rates and severity of dental fluorosis, impacts on thyroid function and performance on IQ tests on all the children. Xiang and coworkers found a statistically significant negative relationship between urinary [6], serum [7], and drinking water [6] fluoride levels and IQ. In the latter study, in which the dose-response relationship was observed, confounding factors of family income, parental education levels, and urine iodine levels were taken into account. The results also showed a dose-response relationship between the percent of children with IQ less than 80 and fluoride levels in drinking water in the high-fluoride village. We combined exposure data from Ref. [6] with additional data from Ref. [10], in which water intake rates and fluoride intakes from food for the two villages were provided, to derive total fluoride exposures for the two village cohorts. We used these exposures shown in **Table 1** to produce **Figure 1**.

Measurements by Xiang et al. [8] of blood-lead levels, and co-exposure to arsenic [9] in the two villages indicated that the decrement in IQ seen in the high-fluoride children was unlikely to have been due to lead or arsenic. The high-fluoride village had lower mean arsenic levels than the low-fluoride village. **Table 2** gives details on the arsenic, lead and iodine measurements in the two villages.

While studies by Xiang et al. [6, 7], Wang et al. [13], Ding et al. [21] and Zhang et al. [14], link lower IQs in children to individualized metrics of fluoride exposure (i.e., urine and serum fluoride), it is not possible at this time to translate directly the dose–responses seen in these studies into safe daily doses. We describe below the techniques we used for that purpose.

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Group	No. of samples*	Water F concentration* (mg/L)	Water F dose† (mg/day)	Total F dose† (mg/day)	IQ
F	290	0.36 ± 0.15	0.45 ± 0.19	0.87 ± 0.19	100.41 ± 13.21
A	9	0.75 ± 0.14	0.93 ± 0.17	1.54 ± 0.17	99.56 ± 14.13
B	42	1.53 ± 0.27	1.90 ± 0.34	2.51 ± 0.33	95.21 ± 12.22‡
C	111	2.46 ± 0.30	3.05 ± 0.37	3.66 ± 0.37	92.19 ± 12.98§
D	52	3.28 ± 0.25	4.07 ± 0.31	4.68 ± 0.31	89.88 ± 11.98§
E	8	4.16 ± 0.22	5.16 ± 0.27	5.77 ± 0.27	78.38 ± 12.68§

*The number of samples in the groups and the water F concentrations are from Xiang et al. [6].

†The water and food fluoride doses are from Xiang et al. [10].

Total fluoride dose (mg F/day): for group F from the low-fluoride village of Xinhuai = water fluoride dose +0.42 mg/day from food; for groups A–E from the high-fluoride village of Wamiao = water fluoride dose +0.61 mg/day from food. The SDs for the mean food fluoride intakes were not reported by group. Compared to group F: ‡$p < 0.05$; §$p < 0.01$.

Values are mean ± SD.

Table 1. Water fluoride (F) concentrations (mg F/L) and doses (mg F/day), total fluoride doses from both water and food (mg F/day), and IQs, in the low-fluoride village of Xinhuai (F) and the high-fluoride village of Wamiao (A–E).

The United States Environmental Protection Agency (USEPA) is in the process [22–24], of developing a new Maximum Contaminant Level Goal (MCLG) for fluoride as recommended by the NRC Committee on Fluoride in Drinking Water [1]. The MCLG is a non-enforceable health-based drinking water goal, and serves as a basis for the development of the enforceable

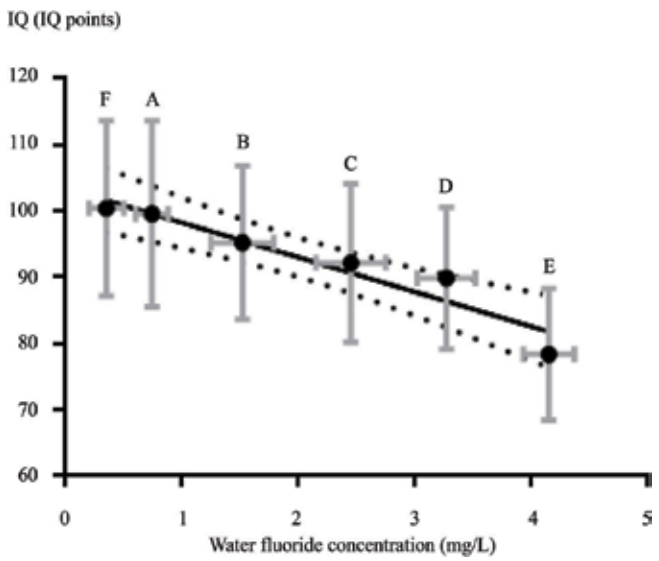

Figure 1. IQ measurements versus water fluoride levels in Wamiao and Xinhuai [6]. The IQ (mean±standard deviation, IQ points) and water fluoride (F) concentrations (mean±SD, mg F/L) in low-F Xinhuai village (F) and high-F Wamiao village (A–E). The letter designations F and A–E correspond to the groups listed in **Table 1**. The values for the IQ and drinking water F concentration are from **Table 8** in Xiang et al. [6]. The dotted curves are the 95% confidence intervals for the best fit linear regression line.

Element	Parameter	High-fluoride village of Wamiao	Low-fluoride village of Xinhuai	p
Arsenic* (μg/L)	n	17	20	
	Mean ± SD	0.24 ± 0.26	16.40 ± 19.11	0.001
	Range	0–0.50	0–48.50	
Iodine† (μg/L)	n	46	40	
	Mean ± SD	280.7 ± 87.2	301.0 ± 92.9	>0.3
	Range	131.3–497.1	148.5–460.9	
Lead‡ (μg/L)	n	71	67	
	Mean ± SD	22.0 ± 13.7	23.6 ± 14.2	>0.48
	Range	1.36–55.0	1.36–61.1	

*The arsenic levels in the drinking water are from Xiang et al. [9].

†The urinary iodine levels are from Xiang et al. [6].

‡The blood-lead levels are from Xiang et al. [8].

Table 2. Levels of the drinking water arsenic (μg/L), the urinary iodine (μg/L), and the blood lead (μg/L) in the children in the high-fluoride village of Wamiao and the low-fluoride village of Xinhuai (n = sample size, values are mean ± standard deviation (SD), and range).

federal standard Maximum Contaminant Level (MCL). The current MCLG is 4 mg/L, which was established to protect against crippling skeletal fluorosis [20]. In order to establish a new MCLG, USEPA must anticipate the adverse effect of fluoride that occurs at the lowest daily dose and then set the MCLG at a level to protect against that effect for everyone, including sensitive subpopulations, with an adequate margin of safety [25].

2. Objective

Our objectives were to address the Broadbent study [5], to estimate a daily dose of fluoride with an adequate margin of safety that would be consistent with the mandate facing USEPA in setting a new MCLG that might prevent reduced IQ in children, including sensitive subpopulations, and to estimate the economic impact of IQ loss among US children.

3. Method

3.1. General

We used two data sets and two risk analysis methods in our risk work. The first data set included the group of 10 studies in [4] that found IQ decrements among children drinking water with 3 mg/L or less fluoride, along with rates of water and food fluoride intakes from [10]. These were used to estimate a lowest observed adverse effect level (LOAEL) for IQ loss. The second data set included IQ measurements corresponding to specific drinking water fluoride levels from [6] along with the water and fluoride in food intake rates cited above, which permitted estimation of daily fluoride doses.

The two risk analysis methods were the lowest-observed-adverse-effect level /no-observed-adverse-effect level (LOAEL)/(NOAEL) and the benchmark dose (BMD) methods, both of which are used by USEPA and both of which include uncertainty factors (UFs) as described below. These risk analysis methods depend upon first estimating from available data either the highest dose that does not result in an observed adverse effect, NOAEL, or in the case of the BMD method, a dose that would result in a specified level of adverse effect. The UFs aim to provide an adequate margin of safety to protect against the adverse effect. They are applied to estimate the NOAEL (in the LOAEL/NOAEL method) and to account for, e.g. inter-individual variability, *in utero* toxicity, severity of the effect, *inter alia* (see below). As used by USEPA, generally no more than three UFs are applied in any analysis, and they are set at 1, 3, or 10, representing, respectively, no need for adjustment, one-half, or one order of magnitude. The daily dose estimated by these methods is known as the Reference Dose (RfD), which is a dose—within one order of magnitude—that can be experienced throughout life without adverse effect. It is normally expressed as mg/kg of body weight per day, mg/kg/day.

We chose instead to express RfD values in units of mg/day for the following reasons. Our analysis was based on data from studies that measured daily intakes of fluoride, reported in mg/day, by children generally aged 8–13 years, most of whom were Chinese. Given published evidence for *in utero* toxicity, discussed below, it is not possible to know at what developmental stage(s) the observed adverse effect was manifested in these children. This makes estimating an RfD in mg/kg/day problematic. Given these considerations, we elected to express RfD values in mg/day that may protect over the entire period from conception through adolescence. Furthermore, we were able to make direct comparison of our results with the estimated daily intakes of US children in mg/day that are presented in Table 7-1 by USEPA [24]. An estimate of an RfD expressed as mg/kg/day is given in **Table 3** below.

3.2. LOAEL/NOAEL method

To avoid over estimating risk, we considered a 3.0 mg/L drinking water fluoride level from Ref. [4] as a Lowest Observed Adverse Effect Concentration, even though at least three other lower concentrations (0.88 mg/L [26]; 1.53 mg/L [6]; and 1.40 mg/L [14]; the latter two with $p < 0.05$ and $p < 0.01$, respectively, from controls) have been associated with loss of IQ. We considered the combined water (1.24 L/day) and food intake rates from [10] (0.50 mg/day, mean of high-fluoride and low-fluoride villages), to be the LOAEL. We used these values because all the work of Xiang et al. was with the same cohort of 512 children, aged 8–13 years, and most of the studies reported by Choi et al. [4] were on children of the same or similar age range and in the same country. (2 of the 10 Choi et al. [4] studies with high-fluoride levels of less than 3 mg/L were from Iran.) We applied three UFs to the LOAEL: one each to estimate the NOAEL, UF 3; to account for inter-individual variability, UF 10; and *in utero* toxicity, UF 3. We chose these UF values because the well-documented effect of neurotoxicity of fluoride does not seem to require higher uncertainty adjustments for LOAEL to NOAEL and for *in utero* toxicity. However, the relatively small number of individuals, primarily Chinese children, on whom we base our work, does merit an uncertainty adjustment of a full order of magnitude for inter-individual variability.

3.3. Benchmark dose method

This method uses a computer program to fit the dose-response data and to determine a dose that results in a specified adverse effect level, known as the Benchmark Response (BMR) or the point of departure (POD). The program also yields the lower 95th confidence limit on the BMD referred to as the BMDL. From this BMDL a NOAEL can be estimated by applying an UF as described above. We used total daily fluoride dose data shown in **Table 2** with USEPA's Benchmark Dose Software [27], setting the BMR at loss of 5 IQ points. Among available BMD models, the linear model showed the best fit with the dose-response data (see **Figure 2**).

We applied UF's for inter-individual variability, and another to account for probable pre-natal toxicity as described above, to the BMDL produced by the program to reach the RfD. For comparison we also ran the program using a BMR of 1 standard deviation (SD) from the mean IQ of the control village, Xinhuai, and we also used a BMR for loss of 1 IQ point.

3.4. Economic impact estimates

Detailed studies on the economic impact of IQ loss associated with exposures to methylmercury, lead, and endocrine-disrupting chemicals have been published by Trasande et al. [28], Attinal and Trasande [29], Bellanger et al. [30], respectively. Based on these studies and

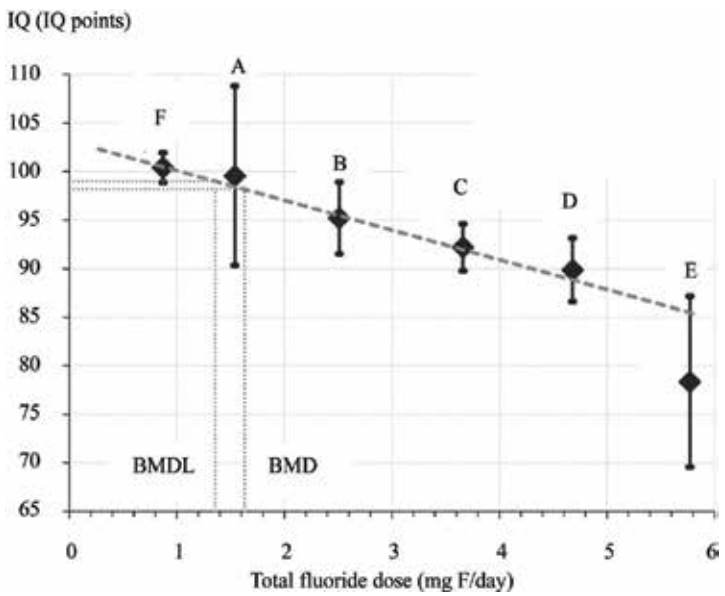

Figure 2. BMD analysis of IQ and total fluoride dose in Wamiao and Xinhuai [6, 10]. The benchmark dose analysis of IQ and the total daily fluoride dose in low-F Xinhuai village (F) and high-F Wamiao village (A–E). The letter designations F and A–E correspond to the groups listed in **Table 1**. The Benchmark Response (BMR) was set at a loss of 5 IQ points. IQ = 103.17 - (3.0675 × total fluoride dose). The error bars are the 95% confidence intervals for IQ. BMDL = Benchmark dose lower-confidence level; BMD = Benchmark dose. The values for the total daily dose of fluoride are from Xiang et al. [6, 10] as noted in the footnote to **Table 1**. The values for the IQ are from **Table 8** in Xiang et al. [6].

our estimated safe levels of exposure to fluoride, we estimated a range of economic losses among US children associated with fluoride exposure. We estimated the economic impact of loss of 1 IQ point on the lifetime income of children in the USA, based on an estimated loss of 1.93% of lifetime income loss for a male and 3.22% loss for a female associated with loss of 1 IQ point [29, 31]. USEPA [31] assigned a value of about $472,000 lifetime income for both males and females, while Trasande et al. [28] assigned values of about $1,000,000 for males and $763,000 for females, both in Year 2000 dollars. Fluoride exposures for US children were taken from USEPA [24] Table 7-1. Assuming all the children in the cohorts described experience fluoride exposures shown in Table 7-1 (but for which we made a correction for drinking water exposure to the mean values given in NRC [1], Table B-4, from the 90th percentile given in Table 7-1) these data lead to an estimate of the economic impact for loss of a single IQ point.

4. Results

Table 3 gives our estimates of fluoride RfDs based on the LOAEL/NOAEL and BMD methodologies. The RfDs range from 0.12 to 0.0090 mg/day for BMDLs set at IQ point losses of 1 SD (from [6]), and 1, respectively.

The RfD based on LOAEL/NOAEL calculations is 0.047 mg/day. We show in **Table 4** results of our BMD analysis of plausible high- and low-fluoride exposures among children in the US based on the same BMD curve used on the Xiang et al. [6] data (**Figure 2**).

RfD method	LOAEL (mg F/day)	RfD (mg F/day)
LOAEL/NOAEL	4.22*	0.047‖
$BMDL_5$†	1.35	0.045**
$BMDL_1$‡	0.27	0.0090**
$BMDL_{1SD}$§	3.58	0.12**

*Calculation of LOAEL with a lowest adverse effect concentration in drinking water of 3.0 mg F/L: fluoride from water: daily water intake 1.24 L/day concentration of fluoride in water 3 mg F/L = 3.72 mg F/day; F from food: 0.50 mg F/day; total F intake from water and food = 4.22 mg F/day.

†$BMDL_5$ for 5 IQ point loss.

‡$BMDL_1$ for 1 IQ point loss.

§$BMDL_{1SD}$ for 13.21 IQ point loss (1 standard deviation from the control mean IQ).

‖Uncertainty factor (UF) usage with LOAEL/NOAEL RfD method: LOAEL to NOAEL: UF = 3; inter-individual variability: UF = 10; *in utero* toxicity: UF = 3.

**Uncertainty factor (UF) usage with BMDL RfD method: inter-individual variability: UF = 10; *in utero* toxicity: UF = 3.

Table 3. Lowest observed adverse effect levels (LOAELs) and reference doses (RfDs) in mg F/day using the lowest observed adverse effect level/ no observed adverse effect level (LOAEL/NOAEL) and the benchmark dose level (BMDL) methods.

Parameter	Estimated total daily F intake in the hypothetical low F exposure group (mg F/day)	Estimated total daily F intake in the hypothetical high F exposure group (mg F/day)	Ratio of the estimated total daily F intake in the high F exposure group to the estimated total daily F intake in the low F exposure groups	Difference between low and high F exposure groups
Total F intake (mg F/day)	0.50	2.0	4.0	1.5 mg F/day
IQ (IQ points)	101.63	97.03		4.6 IQ points

Table 4. The estimated total daily fluoride (F) intakes (mg F/day) of hypothetical low and high F exposure groups of US children, the ratio of the estimated total daily F intake in the high F exposure group to the estimated total daily F intake in the low F exposure group, and estimations of the IQs in these groups using the benchmark dose (BMD) method of analysis.

5. Discussion

Table 4 indicates that the effect of fluoride on IQ is quite large, with a predicted mean 5 IQ point loss when going from a dose of 0.5 to 2.0 mg/day, which is an exposure range one might expect when comparing individuals in the USA with low total intake to those with higher total intake. However, when comparing a fluoridated area of the USA to an un-fluoridated area it would be hard to discern a mean IQ difference, because of the multiple sources of fluoride intake besides drinking water (**Table 5**). These sources greatly reduce the contrast in total fluoride intake between fluoridated and un-fluoridated areas. A very high hurdle is thus created to gaining useful information in the USA, as it was in the New Zealand study [5], via a large, long-range longitudinal epidemiological study of fluoride and IQ.

Reference number for the values for the lifetime economic loss/loss of one IQ point	Lifetime economic loss in year 2000 dollars ($) for the loss of one IQ point for various groups of children			
	An existing cohort*	A birth cohort*	1 male	1 female
	n = 74,300,000	n = 4,000,000	n = 1	n = 1
[26]†	$896 billion	$48.2 billion	$9110	$15,200
[23]‡	$1650 billion	$88.7 billion	$19,900	$24,600

*The existing and birth cohort sizes are based on values from Ref. [47].

†The lifetime economic loss/loss of one IQ point is based on values from Ref. [31].

‡The lifetime economic loss/loss of one IQ point is based on values from Ref. [23].

Table 5. The lifetime economic loss, in year 2000 dollars ($), for the loss of one IQ point for groups of children consisting of an existing cohort (n = 74,300,000), a birth cohort (n = 4,000,000), 1 male, and 1 female.

In any event, as **Table 4** indicates, based on the dose-response seen in [6], the implication for US children appears to be that children whose fluoride exposures are held to a minimum, e.g. 0.5 mg/day or less, may have as much as a 4 or 5 point IQ advantage, or more, over children whose exposures are greater than 2 mg/day, all other factors affecting IQ being equal.

Table 7-1 from USEPA [24] shows the total fluoride intakes from all sources of exposure by age grouping in mg/day. Based on that table and other data from USEPA [24] and NRC [1] Table B-4, current average mean fluoride exposures for US children range from about 0.80 to about 1.65 mg/day. These doses are 17–35 times higher than our higher estimated RfD. At the 90th percentile of water intake, the total fluoride doses for US children are 25–60 times higher than our higher RfD. These data imply that at present the risk of IQ loss among children in the US is high.

While sources of fluoride cited in Table 7-1 USEPA [24] exceed the fluoride levels that we estimate would be protective for all children, a natural source of fluoride does not. In general, fluoride levels found in human breast milk are approximately 0.004 mg/L, Ekstrand [32], which result in daily doses of ca. 0.002–0.004 mg/day USEPA [33]. These doses are well below our estimated RfD, including the value we obtained by BMD analysis using a 1 point IQ loss BMR. However, it should be noted that high breast milk fluoride levels, mean 0.550 mg F/L, have been reported from Koohbanan, Iran, altitude >2000 m, [34] and the possible role of an altitude effect in this has been queried [35].

While the breast ordinarily provides protection from the mother's serum fluoride levels [32], the placenta does not. Fluoride readily crosses the placenta and, in general, average cord blood concentrations are approximately 60% of maternal serum concentrations of fluoride [36]. Evidence that fluoride affects neural development *in utero* has been shown in a number of human studies. For example, He [37] found that pre-natal fluoride toxicity occurs in humans, manifested in alteration in the density of neurons and in the number of undifferentiated neurons observed in therapeutically aborted fetuses. Yu et al. [38] found reduced synthesis of neurotransmitters and a decrease in the density and function of their receptors in brains of aborted fetuses in an endemic fluorosis area of China compared to similar fetuses in a non-endemic fluorosis area. Dong et al. [39] found differences in amino acid and monoamine neurotransmitter content in brains of aborted fetuses from an endemic fluorosis area of China compared with those from a non-fluorosis area. Both bone and brain tissues of these fetuses showed statistically significantly higher fluoride levels from the fluorosis area than from the control area. Du et al. [40] reported in detail on the adverse changes in neuron development found in brain tissue from fetuses from endemic fluorosis areas of China (fluoride levels 0.28 ± 0.14 μg/g) compared to similar tissues from non-endemic areas (fluoride level 0.19 ± 0.06 μg/g) ($p < 0.05$). Mullenix et al. [41] showed that pregnant rats dosed with fluoride at a level that produced serum fluoride levels equivalent to those observed in humans who consumed drinking water at the current MCLG concentration of 4 mg/L gave birth to pups displaying lifelong neurological impairment. Finally, Choi et al. [42] discussed the fact that, "…systemic exposure should not be so high as to impair children's neurodevelopment especially during the highly vulnerable windows of brain development *in utero* and during infancy…" In this regard, the fluoride intake levels that the mothers of the subject children

from the Choi et al. studies [4, 42] and Xiang et al. studies [6, 10] experienced may have played a part in the reported IQ losses. For this reason, the RfD values we derived may have some value for protection of fetuses carried by pregnant women as well as for the children in infancy that they subsequently deliver.

We relied on data from the meta-analysis [4] that employed well-documented selection criteria for the subject studies used in the analysis, and that provided "evidence supporting a statistically significant association between the risk factor" (fluoride exposure) and lowered IQ among higher fluoride exposed children. In so doing we conformed to the recommendation of Bellinger [43] regarding use of meta-analyses in assessments like ours. The Choi et al. [4] meta-analysis found an average decrement of about 7 IQ points in the higher fluoride exposed groups, and the ten studies from it on which we based our use of 3 mg/L as the adverse effect concentration showed an average decrement of 8 points. Based on our RfD findings, it is reasonable to suspect that some children in the USA have experienced IQ loss from pre- and post-natal fluoride exposures.

We calculated RfD values for the two extreme drinking water fluoride exposures in publications cited in Ref. [4]. Wang et al. [13] showed statistically significant IQ loss in children at a mean drinking water fluoride level of 8.3 mg/L. Using the same LOAEL/NOAEL methodology and the same water and food intake assumptions as above, we derived a RfD of 0.12 mg/day. Lin et al. [26] showed a statistically significant IQ loss in an area with low-iodine intakes with a fluoride water level of 0.88 mg/L, leading to an RfD of 0.018 mg/day. The latter study is significant because the Safe Drinking Water Act [25] stipulates that the whole population, including sensitive subgroups, must be protected by the MCLG for fluoride. In the 2007–2008 National Health and Nutritional Examination Survey, Caldwell et al. [19] found that about 5% of children aged 6–11 years had a urinary iodine concentration of <50 μg/L. Urinary iodine levels 20–49 μg/L indicate moderate iodine deficiency and levels <20 μg/L show severe deficiency [44]. Thousands of US children fall into this sensitive subgroup of iodine deficiency. Since USEPA [24] apparently intends to protect 99.5% of US children from severe dental fluorosis with a new MCLG, it is not unreasonable to expect that USEPA would take iodine insufficiency into account as a risk factor for IQ loss from fluoride as well.

In a population of 320 million the population level impact of an average 5 IQ point loss, beyond purely dollars of income loss, is a reduction of about 4 million people with IQ >130 and an increase of almost as many people with IQ < 70 [45].

6. Limitations

In general, our RfD work is based on a limited amount of quantitative data, most of which is from Chinese studies, most of which were of ecological design. Unfortunately, we were unable to find any data on human intellectual performance as a function of fluoride exposures in the USA. Nor were there studies, other than those by the Xiang research groups, which provided any useful dose–response information. While there is growing interest in the USA in this area of research, there are significant impediments to such work as mentioned above.

In estimating RfD values we used mean water consumption rates, except as noted, and mean IQ measurements that were derived from different testing methods, recognizing the limitations of these uses and those inherent in ecological studies generally. The data we used for the food component in estimating total fluoride intakes were also mean values from one study that were not accompanied by standard deviations. They were, however, somewhat higher than the values for children's food fluoride exposures in the USA. This indicates that we used a conservatively high-fluoride dose to estimate the adverse effect level from those studies. Inasmuch as the timing effect of fluoride exposure on neurodevelopment is not precisely known, these age-variable mean consumption rates may introduce some error. Further, it may be that fluoride exposures that the pregnant mother experiences may at least partially influence the outcome for the child.

The RfDs we estimated were derived from data on primarily Chinese children of similar age and body mass to children in the USA, for whom these safe levels are intended. Finally, use of mean measured IQ levels cannot speak to the experience of individual children for a variety of reasons, and Choi et al. [4] point out this limitation. While Choi et al. [4, 42] urge caution in using their results to determine an exposure limit, we feel we have been cautious, and that simply ignoring the available dose-response information amid the substantial body of evidence of developmental neurotoxicity could result in policies that are insufficiently protective of public health. Finally, based on available data, which do not provide sufficient information to assess at what stage the adverse effects of fluoride on neural development occur, one cannot be certain that there is any safe daily dose of fluoride that would prevent developmental neurotoxicity.

Limitations inherent to both the BMD and LOAEL/NOAEL methods, including the quantity and quality of underlying research and the number and values selected for UFs apply to our use of those methods for determining RfDs. Clearly, it would have been useful to have a more robust data set on which to base our risk analysis, but waiting for more such data that are unlikely to be developed in the near future did not seem reasonable to us.

7. Conclusions

The information now available supports a reasonable conclusion that exposure of the developing brain to fluoride should be minimized, and that economic losses associated with lower IQ's may be quite large. While Choi et al. [42] also caution against systemic exposures to "high levels" of fluoride, the requirement of the Safe Drinking Water Act to protect all children, including those with special sensitivities and those *in utero,* against developmental neurotoxicity makes it imperative to be conservative in defining the term, "high level." We believe our analysis provides some insight on this definition.

Because it is not clear what stage(s) of development is/are sensitive to fluoride toxicity, well-funded research into this effect should be a priority. If sufficient exposure information were to be gathered, it would be useful in identifying where and among whom the greatest risk for IQ loss exists. The work of Zhang et al. [14] and iodine data reported in [19] are germane to

this point. Meanwhile, based on current information, implementation of protective standards and policies seems warranted and should not be postponed while more research is done. The amount of consistently observed adverse effects on neurological development reported by multiple research groups world-wide, which culminated in the addition of fluoride by Grandjean and Landrigan [46] to their list of known developmental neurotoxicants, and the imminent publication of a health-based fluoride drinking water standard in the USA makes addressing extant data mandatory sooner rather than later.

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References

[1] National Research Council, Committee on Fluoride in Drinking Water. Fluoride in Drinking Water: A Scientific Review of USEPA Standards. Washington, DC, USA: National Academies Press; 2006. Pdf available from National Academies Press at: http://www.nap.edu/catalog/11571.html. Accessed 16-06-2016

[2] Rocha-Amador D, Navarro ME, Carrizales L, Morales R, Calderon J. Decreased intelligence in children and exposure to fluoride and arsenic in drinking water. Cad Saúdé Publica. 2007;**23**(suppl.4. Available at: http://dx.doi.org/10.1590/20102-311X2007001600018) Accessed 16-10-2016

[3] Am Env Health Studies Proj. Fluoride Toxicity Data Base. Available at: http://fluoride-alert.org/?s=neurotoxicity Accessed Sept. 19, 2014

[4] Choi AL, Sun G, Zhang Y, Grandjean P. Developmental fluoride neurotoxicity: A systematic review and meta-analysis. Environmental Health Perspectives. 2012;**120**:1362-1368. Available at: http://dx.doi:10.1016/j.ntt.2014.11.001 Accessed Oct. 13, 2012

[5] Broadbent JM, Thomson WM, Ramkha S, Moffitt TE, Zeng J, Page LAF, et al. Community water fluoridation and intelligence: Prospective study in New Zealand. American Journal of Public Health. 2015;**105**:72-76

[6] Xiang Q, Liang Y, Chen L, Wang C, Chen B, Chen X, et al. Effect of fluoride in drinking water on children's intelligence. Fluoride. 2003;**36**:84-94

[7] Xiang Q, Liang Y, Chen B, Chen L. Analysis of children's serum fluoride levels in relation to intelligence scores in a high and low fluoride water village in China. Fluoride. 2011;**44**:191-194

[8] Xiang Q, Liang Y, Zhou M, Zang H. Blood lead of children in Wamiao-Xinhuai intelligence study. Fluoride. 2003;**36**:198-199

[9] Xiang Q, Wang Y, Yang M, Zhang M, Xu Y. Level of fluoride and arsenic in household shallow well water in Wamiao and Xinhuai villages in Jiangsu Province, China. Fluoride. 2013;**46**:192-197

[10] Xiang Q, Zhou M, Wu M, Zhou X, Lin L, Huang J. Relationships between daily total fluoride intake and dental fluorosis and dental caries. Journal of Nanjing Medical University. 2009;**23**:33-39

[11] Menkes DB, Thiessen K, Williams J. Health effects of water fluoridation-how "effectively settled" is the science [letter]. New Zealand Medical Journal. 2014;**127**(1407):6394. Available at: http://www.nzma.org.nz/journal/read-the-journal/all-issues/2010-2019/2014/vol-127-no-1407/6394 Accessed 16-10-2016

[12] Malin AJ, Till C. Exposure to fluoridated water and attention deficit hyperactivity disorder prevalence among children in the United States: an ecological association. Environmental Health. 2015;**14**:17. Available at: http://dx.doi:10.1186/s12940-015-0003-1 Accessed Mar. 1, 2015. Published online 27 Feb. 2015

[13] Wang SX, Wang ZH, Cheng XT, Li J, Sang ZP, Zhang XD, et al. Arsenic and fluoride exposure in drinking water: children's IQ and growth in Shanyin County, Shanxi Province, China. Environmental Health Perspectives. 2007;**115**:643-647

[14] Zhang S, Zhang X, Liu H, Qu W, Guan Z, Zeng Q, Jiang C, et al. Modifying effect of *COMT* gene polymorphism and a predictive role for proteomics analysis in children's intelligence in endemic fluorosis area in Tianjin, China. Toxicological Sciences. Published online Jan. 1, 2015. Available at: http://dx.doi:10.1093/toxsci/kfu311. Accessed Jan. 20, 2015

[15] Peckham S, Lowery D, Spencer S. Are fluoride levels in drinking water associated with hypothyroidism prevalence in England? A large observational study of GP practice data and fluoride levels in drinking water. Epidemiology and Community Health. 2015;**69**(7):619-624. DOI: 10.1136/jech-2014-204971. Published online 24 Feb. 2015

[16] Klein RZ, Sargent JD, Larsen PR, Waibren SE, Haddow JE, Mitchell ML. Relation of severity of maternal hypothyroidism to cognitive development of offspring. Journal of Medical Screening. 2001;**8**(1):18-20

[17] World Health Organization, United Nations Children's Fund & International Council for the Control of Iodine Deficiency Disorders. Assessment of Iodine Deficiency Disorders and Monitoring their Elimination. 3rd ed. Geneva, Switzerland: WHO; 2007 in: NIH 2011

[18] WHO Secretariat, Andersson M, de Benoist B, Delange F, Zupan J. Prevention and control of iodine deficiency in pregnant and lactating women and children less than 2-years-old: Conclusions and recommendations of the technical consultation. Public Health Nutrition. 2007;**10**(12A):1606-1611. [PubMed abstract] in: NIH 2011

[19] Caldwell KL, Makhmudov A, Ely E, Jones RL, Wang RY. Iodine status of the U.S. population, National Health and Nutrition Examination Survey, 2005-2006 and 2007-2008. Thyroid. 2011;**21**:419-427 https://doi.org/10.1089/thy.2010.0077 Accessed 04-05-2015

[20] U.S. Environmental Protection Agency. National Primary Drinking Water Regulations: Fluoride. Final rule. 50 Federal Register 47142. November 14, 1985; 51 Federal Register 11396. Apr. 2, 1986

[21] Ding Y, Gao Y, Sun H, Han H, Wang W, Ji X, et al. The relationship between low levels of urine fluoride on children's intelligence and dental fluorosis in endemic fluorosis areas in Hulunbuir, Inner Mongolia, China. Journal of Hazardous Materials. 2011;**186**:1942-1946. Available at: http://dx.doi:10.1016/j.mazmat.2010.12.097 Accessed Apr. 15, 2014

[22] US Environmental Protection Agency. National Primary Drinking Water Regulations: USEPA's Review of Existing Drinking Water Standards and Request for Public Comment. 67 Federal Register 19069. April 17, 2002 et seq; U.S. USEPA, Six-year review chemical contaminants. Health effects technical support document. USEPA 822-R-03-008, June 2003

[23] U.S. Environmental Protection Agency. Fluoride: Dose-Response Analysis for Non-Cancer Effects. Health and Ecological Effects Division, Office of Water; December 2010. Available at: http://water.USEPA.gov/action/advisories/drinking/upload/Fluoride_dose_response.pdf Accessed Sept. 2, 2014

[24] U.S. Environmental Protection Agency. Fluoride: Exposure and Relative Source Contribution Analysis. December 2010. Available at: http://water.USEPA.gov/action/advisories/drinking/upload/Fluoridereport.pdf. Accessed Sept. 2, 2014

[25] Safe Drinking Water Act (As amended through P.L.107-377, December 2002). In: Title XIV of the public health service act (the safe drinking water act). Title XIV. Section 1412(b)(4) goals and standards. Available at: http://www.epw.senate.gov/sdwa.pdf. Accessed Sept. 19, 2014

[26] Lin FF, Aihaiti, Zhao HX, Lin J, Jiang JY, Maimati A. The relationship of a low-iodine and high-fluoride environment to subclinical cretinism in Xinjiang (in Chinese). Bulletin of Endemic Diseases; **6**:62-67. Translation available at: http://www.fluoridealert.org/wp-content/uploads/lin-19912.pdf Accessed Dec. 10, 2014

[27] U.S. Environmental Protection Agency. Benchmark dose software (BMDS). Available at: http://www.USEPA.gov/nceawww1/bmds/index.html Accessed 20-09-2014

[28] Trasande L, Landrigan PJ, Schecter C. Public health and economic consequences of methyl mercury toxicity to the developing brain. Environmental Health Perspectives. 2005;**113**:590-596. Available at: http://www.dx.doi:10.1289/ehp.7743 Accessed Nov. 24, 2014

[29] Attinal TM, Trasande L. Economic costs of childhood lead exposure in low- and middle-income countries. Environmental Health Perspectives. 2013;**121**:1097-1102. Available at: http://dx.doi.org/10.1289/ehp.1206424 Accessed Jan. 10, 2016

[30] Bellanger M, Demeneix B, Grandjean P, Zoeller RT, Trasande L. Neurobehavioral deficits, diseases, and associated costs of exposure to endocrine-disrupting chemicals in the European Union. The Journal of Clinical Endocrinology and Metabolism. 2015;**100**(4):1256-1266. Published online 2015 Mar 5. Available at http://www.dx.doi.10.1210/jc.2014-4324 Accessed 10-01-2016

[31] U.S. Environmental Protection Agency, Griffiths C, McGartland A, Miller M. A note on Trasande et al., "public health and economic consequences of methylmercury toxicity to the developing brain". In: Working Paper # 06-02. National Center for Environmental Economics. Available at: http://www.USEPA.gov/ee/USEPA/eed.nsf/44a8be610f6c5f0885256e46007b104e/dd32a21a7da2bdf38525715500485642/$file/2006-02.pdf; April, 2006 Accessed 04-04-2015

[32] Ekstrand J. No evidence of transfer of fluoride from plasma to breast milk. British Medical Journal. 1981;**283**:761-762

[33] U.S. Environmental Protection Agency. Child-Specific Exposure Factors Handbook. table 15-1. Available at: http://cfpub.USEPA.gov/ncea/cfm/recordisplay.cfm?deid=199243 Accessed 06-03-2015

[34] Poureslami H, Khazaeli P, Mahvi AH, Poureslami K, Poureslami P, Haghani J, Aghaei M. Fluoride level in the breast milk in Koohbanan, a city with endemic dental fluorosis. Fluoride. 2016;**49**(4 Pt 2):485-494

[35] Spittle B. Fluoride in human breast milk [editorial]. Fluoride. 2016;**49**(4 Pt2):471

[36] National Research Council. Fluoride in Drinking Water: A Scientific Review of USEPA Standards. Washington, DC, USA: National Academies Press; 2006. p. 193. Available at: http://www.nap.edu/catalog/11571.html Accessed 16-10-2016

[37] He H. The effect of fluoride on the fetus. Chinese Journal of Control of Endemic Diseases. 1989:136 Translated report in Fluoride 2008;**41**:321-326

[38] Yu Y, Yang W, Dong Z, Wan C, Zhang J, Liu J, et al. Neurotransmitter and receptor changes in the brains of fetuses from areas of endemic fluorosis. Chinese Journal of Endemiology. 1996;**15**(5):257-259 Translated report in Fluoride 2008;**41**:134-138

[39] Dong Z, Wan C, Zhang X, Lui J. Determination of the contents of amino acids and monoamine neurotransmitters in fetal brains from a fluorosis endemic area. Journal of Guiyang Medical College. 1997;**18**:241-245 Translation available at: http://fluoridealert.org/wp-content/uploads/dong-1993.pdf

[40] Du L, Wan C, Cao X, Liu J. The effect of fluorine on the developing brain. Chinese Journal of Pathology. 1992;**21**:218-220 Translated report in Fluoride 2008;**41**:327-330

[41] Mullenix PJ, Denbesten PK, Schunior A, Kernan WJ. Neurotoxicology of sodium fluoride in rats. Neurotoxicology and Teratology. 1995;**17**:169-177

[42] Choi AL, Zhang Y, Sun G, Bellinger DC, Wang K, Yang XJ, et al. Association of lifetime exposure to fluoride and cognitive functions in Chinese children: A pilot study. Neurotoxicology and Teratology. 2015;**47**:96-101 http://dx.doi.org/10.1016/j.ntt.2014.11.001 Accessed Feb. 22, 2015

[43] Bellinger DCA. strategy for comparing contributions of environmental chemicals and other risk factors to neurodevelopment of children. Environmental Health Perspectives. 2012;**120**:501-507

[44] Thyroid/Iodine Deficiency. Available at: http://emedicine.medscape.com/article/122714-overview Accessed Sept. 19, 2015

[45] Weiss B. A 5 point loss in IQ. In: Colborn T, Dumanoski D, Myers JP, editors. Our Stolen Future. New York: E.P. Dutton; 1996. ISBN 978-0-525-93982-5 p. 236

[46] Grandjean P, Landrigan PJ. Neurobehavioural effects of developmental neurotoxicity. Lancet Neurology. 2014;**13**:330-338

[47] Childstats.gov 2015s.gov. 2015. Available from: http://www.childstats.gov/pdf/ac2014/ac_14.pdf. Accessed 18-03-2015

Chapter 8

Targeting Acid-Sensing Ion Channels by Peptide Toxins

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Additional information is available at the end of the chapter

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Abstract

Acid-sensing ion channels (ASICs) are proton-gated ion channels that are highly expressed in the nervous system and play important roles in physiological and pathological conditions. They are also expressed in non-neuronal tissues with different functions. The ASICs rapidly respond to a reduction in extracellular pH with an inward current that is quickly inactivated despite the continuous presence of protons. Recently, protons have been identified as neurotransmitters in the brain. Until now, six different isoforms (ASIC1a, 1b, 2a, 2b, 3 and 4) in rodents have been discovered and they can be assembled into homotrimers or heterotrimers to form an ion channel. Peptide toxins targeting ASICs have been found from the venoms of spider Psalmotoxin-1 (PcTx1), sea anemones (APETx2 and PhcrTx1) and snakes (MitTx and mambalgins). They reveal different pharmacological properties and are selective blockers of ASICs, except for MitTx, which is a potent activator of ASICs. In this mini review, the structure, pharmacology and effects of peptide toxins on ASICs will be introduced and their therapeutic potentials for neurological and psychological diseases will be discussed.

Keywords: acid-sensing ion channels, peptide neurotoxins, pain, stroke, depression, neuron

1. Introduction

With great interests in venom toxins, scientists are extremely involved and enthusiastic about this area of research, as applications of these venoms for drugs could bring about a greater understanding of human diseases, potentially changing and advancing human healthcare [61, 65]. Venoms of species like spiders, sea anemones and snakes have been found to target ion channels with highly therapeutic potentials as drug candidates [17, 38]. To explore structure-function, gating mechanisms and tissue localization of many ion channels, animal venom toxins were important pharmacological tools in the ion channel field [28]. Certain peptides even lead to clinical development and venom-based drugs, such as ziconotide, which is an inhibitor of neuronal

voltage-gated calcium channels isolated from *Conus magus*, designed for patients with intractable pain who fail to respond to other drugs [57, 66].

Recently, protons have been identified as neurotransmitters in the brain [26]. One of the candidate targets for proton sensing is called "acid-sensing ion channels" (ASICs). Three decades ago, the proton-activated inward currents were discovered and recorded in neurons isolated from rat spinal ganglia and from the ganglion of trigeminal nerve by the pioneer Krishtal and Pidoplichko [48, 49]. Twenty years ago, Waldmann et al. first cloned the ASICs [80]. ASICs are widely expressed in the nervous system with high density [1, 62, 80]. Molecular cloning of ASICs has identified four genes (*ACCN1–4*) encoding at least six ASIC subunits in rodents (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4) [35, 84]. Structurally, each ASIC subunit consists of 500–560 amino acids with a simple topology: two transmembrane domains, large extracellular loop (370 amino acids) and short intracellular N- and C-terminals (35–90 amino acids). The structure of ASICs is different from traditional ligand-gated G-protein couple receptor, which has seven transmembrane domains. ASICs can form functional ion channels structurally appearing as trimeric complexes of these subunits [44], which form both homomeric and heteromeric channels with different electrophysiological and pharmacological properties [3, 11, 37, 45, 51, 67, 71]. Among all the ASIC subunits, the ASIC2b and ASIC4 subunits do not form functional homomeric proton-gated ion channels by themselves, but they can associate with other ASIC subunits to reveal new pharmacological properties on the heteromeric channels [21, 51, 67].

ASICs are mainly expressed in the central and peripheral nervous systems, chiefly found in neurons [80, 82]. In central nervous system, ASICs contributed to several physiological and pathological conditions, such as learning and memory, fear conditioning, pain, chemoreception, ischemia, seizures, drug addiction and neuroinflammation, where extracellular acidification occurs [5, 9, 12, 82–84]. More importantly, ASICs are involved in synaptic physiology and are neurotransmitter receptors critical for amygdala-dependent learning and memory [26]. In peripheral sensory neurons such as dorsal root ganglia (DRG), ASIC1, 2 and 3 are found. During pathological condition such as inflammation, tumors or wounds, peripheral tissue acidosis associated with pain occurs. ASICs are of particular interest because they are profoundly sensitive to moderate acidifications [18]. They are more sensitive than transient receptor potential vanilloid 1 (TRPV1), another ion channel activated by protons, capsaicin and heat in nociceptive neurons. ASICs can produce sustained depolarizing currents upon prolonged tissue acidification compatible with the detection of non-adapting pain [18]. ASIC currents and/or transcripts have also been found in glia, smooth muscle cells, lung epithelial cells, immune cells, urothelial cells, adipose cells, joint cells and osteoclasts, indicating that ASICs likely play a role in non-neuronal cells as well [18, 32, 35, 50, 59, 70, 86]. The review regarding the effects of peptide toxins on ASICs has also been discussed by previous publications [4, 5, 9, 10, 12, 17].

2. Targeting ASICs by peptide toxins

2.1. Psalmotoxin-1 (PcTx1)

Among all the peptide toxins, PcTx1 is the first peptide discovered for the ASICs. PcTx1 was identified from venom of the South American tarantula *Psalmopoeus cambridgei* [30, 31]. It is a

potent and selective inhibitor for both homomeric ASIC1a and heteromeric ASIC1a/2b channels [31, 67]. Structurally, this toxin has 40 amino acids crosslinked by three disulfide bridges [31]. Pharmacologically, the IC_{50} of PcTx1 is 0.9 nM for homomeric ASIC1a channels [30, 31] and 2.6 nM for heteromeric ASIC1a/2b channels [67] in *Xenopus oocytes* expressed homomeric ASIC1a or heteromeric ASIC1a/2b channels. In our previous studies, PcTx1 at a concentration of 10 nM significantly inhibits ASIC currents in majority of cultured striatal and cortical neurons, respectively [45, 89]. At concentrations that effectively inhibit the homomeric ASIC1a current, it has no effect on the currents mediated by other configurations of ASICs such as heteromeric ASIC1a/2a channels [31] or known voltage-gated Na^+, K^+, Ca^{2+} channels as well as several ligand-gated ion channels [89]. Unlike amiloride, which is a blocker of epithelial sodium channel and directly blocks the ASICs, PcTx1 acts as a gating modifier [9, 35]. PcTx1 shifts the channel from its resting state toward the inactivated state by increasing its apparent affinity for protons [5, 17].

Purified PcTx1 or venom toxin was the first peptide used to explore the function of ASICs in neurological, psychological and other diseases [5]. Our previous studies have shown that PcTx1 reveals neuroprotective effects on mouse cultured cortical neurons subjected to extracellular acidosis as well as oxygen and glucose deprivation [88, 89]. In a rodent experimental stroke model (middle cerebral artery occlusion), central injection of venom toxin or PcTx1 significantly reduces the infarct volume by 60% and the protection by PcTx1 treatment lasts 1 week [60, 89]. Consistent with our findings, similar effect by application of PcTx1 was also found in a model of traumatic spinal cord injury in rats [39]. Venom toxin also shows certain protection in a mouse model of multiple sclerosis associated with axonal degeneration [33] as well as in the mouse MPTP model of Parkinson's disease [2]. Moreover, PcTx1 decreases the acidosis-mediated cell death in cultured retinal ganglion cells [74]. Collectively, all the results support that PcTx1 might be a potential therapeutic agent for neurological disease [9, 12, 81, 87, 88].

ASIC1a is highly expressed in the amygdala, a brain region critical for fear, arousal and emotions [82, 84, 85]. Central injection of venom toxin reduces mouse innate fearing [14, 16], mouse depression-related behavior [15] and stress-induced elevation in core body temperature of mice [29]. The mechanisms of fear reduction, antidepressant and anxiolytic effects by PcTx1 are likely mediated by inhibition of ASIC1a-containing channels in the amygdala.

PcTx1 has also been used to study pain modulation in rodents [54]. Treatment by PcTx1 was shown to induce a potent analgesic effect in acute pain, inflammatory and neuropathic pain models in mice [54].

ASICs are involved in the central chemoreception [40, 71, 72]. Central injection of PcTx1 in the lateral hypothalamus (LH), nucleus of the solitary tract (NTS) and rostral ventrolateral medulla (RVLM) inhibits the acid-induced stimulating effect on respiration [40, 71, 72]. Thus, ASICs in the LH, NTS and RVLM contribute to central regulation of respiration.

ASICs are also expressed in non-neuronal tissue, including but not limited to smooth muscle cells (VSMC) from arteries, where they might play a role in mechanotransduction of the myogenic response and VSMC migration [25]. ASIC currents recorded in acutely dissociated mice cerebral artery smooth muscle cells are potentiated by PcTx1 in majority of the cells [13]. PcTx1 also reduces store-operated calcium entry in VSMCs in rat pulmonary arteries. By using PcTx1, ASIC1a-containing channels are involved in the vascular mechanotransduction.

PcTx1 itself cannot cross the blood-brain barrier. Therefore, the critical importance is how to deliver the PcTx1 to its correlated damaged specific brain region and to search a small molecule with similar effect as PcTx1 [9].

2.2. APETx2

The peptide toxin APETx2 was isolated from sea anemones (*Anthopleura elegantissima*) and is a selective inhibitor for ASIC3 and ASIC3-containing channels [22]. Structurally, APETx2 contains 42 amino acids crosslinked by three disulfide bonds, a compact disulfide-bonded core with a four-stranded beta-sheet. APETx2 possesses the disulfide-rich all-beta structural family of peptide toxins usually seen in animal venoms. Pharmacologically, APETx2 inhibits both homomeric ASIC3 channels and heteromeric ASIC3-containing channels in heterologous expression systems as well as primary cultures of sensory neurons in rodents. It inhibits the transient component of ASIC3 currents with an IC_{50} of 63 nM, without affecting sustained component of ASIC3 currents [22]. However, the affinity of this particular ASIC3 inhibitor is reduced when ASIC3 is associated with other ASIC subunits [22]. For instance, the IC_{50} for ASIC3/ASIC2b is about 117 nM, whereas the IC_{50} for ASIC3/ASIC1a is around 2 μM [22]. By acting at this external side, APETx2 directly inhibits the ASIC3 channel, and it does not modify the channel unitary conductance [5].

ASIC3 and ASIC3-containing channels are widely expressed in peripheral sensory neurons and play a critical role in pain modulation [8]. During chronic inflammation, the expression level of ASIC3 was upregulated in rat sensory neurons [52, 53, 77], which might be critical for the sensitization of cutaneous nociceptors during inflammation. Consistent with these findings, a reduction in pH in the skin of human volunteers was involved in non-adapting pain [73], and this cutaneous acid-induced pain is largely mediated by ASIC channels, because it is inhibited by amiloride [46, 56, 76]. Additionally, the non-amiloride ASIC blocker, A-317567 exhibits distinct in vitro and in vivo activities over amiloride [27]. Furthermore, by using APETx2, ASIC3 was identified as a sensor of cutaneous acidic pain and postoperative pain and as an integrator of molecular signals released during inflammation in rat, where it is involved in primary thermal hyperalgesia [18–20]. In correlation with this result, local peripheral application of APETx2 was found to attenuate mechanical hypersensitivity in a cutaneous inflammatory pain rat model [47].

ASIC3 is mainly expressed in small muscle afferents in rat [19, 58] and in more than 30% of sensory neurons innervating the knee joint in mouse [42]. The expression level of ASIC3 in sensory neurons is enhanced in models of muscle inflammation [79] and acute arthritis [42] in mice. The application of APETx2, in comparison with ASIC3 knockout and knockdown mice, revealed a critical role for ASIC3 in the generation of secondary mechanical hyperalgesia associated with central sensitization achieved in a mouse model of non-inflammatory muscular pain triggered by repeated acid injections into the muscle [63, 68] and in a mouse model of joint inflammation [41]. Consistent with these findings, peripheral application of APETx2 was also found to decrease mechanical hypersensitivity in a non-inflammatory muscular pain in rat [47]. Furthermore, ASIC3 is also involved in the development of primary cutaneous mechanical hyperalgesia induced by muscle inflammation [69, 78]. In a rat model

of osteoarthritis, continuous intra-articular injections of APETx2 reduced pain-related behavior and secondary mechanical hyperalgesia [43]. An increase in ASIC3 expression was also seen in afferent sensory neurons of the knee joint [43].

APETx2 significantly reduces the exercise pressor reflex mediated by contracting skeletal muscle in rodents [36, 55, 75]. This is supported by the expression of ASIC3 in muscle metaboreceptors [58]. By using ASIC3 knockout mice, researchers have found minor changes in normal cutaneous mechanical sensitivity [8, 63], whereas other studies did not reveal a significant contribution to mechanosensory function [24]. By using selective inhibitor of ASIC3, ASIC3 has been shown to be a neuronal sensor for the skin vasodilation response to direct pressure in both humans and rodents and for skin protection against pressure ulcers in mice [34]. Thus, APETx2 reduces local vascular tone control through blockade of ASIC3 or ASIC3-containing channels.

2.3. Mambalgins

The two peptides of mambalgins (mambalgin-1 and mambalgin-2) were recently found from the venom of the snake *Dendroaspis polylepis polylepis* [23]. Structurally, these two toxins contain 57 amino acids and include eight cysteines linked by four disulfide bridges. Pharmacologically, mambalgins inhibit ASIC-like currents in cultured neurons of hippocampus and spinal cord. Furthermore, mambalgins inhibit homomeric ASIC1a, 1b, heteromeric ASIC1a/2a, 1a/2b and 1a/1b channels with IC_{50} between 50 and 200 nM. Functionally, mambalgins reveal analgesic effects *in vivo* in models of acute and inflammatory pain through either inhibition of ASIC1a and ASIC1a/2a channels in central nervous system or inhibition of ASIC1b channels in peripheral nervous system [5, 23]. Interestingly, the central analgesic effect of mambalgins revealed strong effect similar to morphine but produces less unwanted side effects [4, 23]. Further studies are needed to explore the cellular and molecular mechanisms responsible for such pain pathways, but brain ASICs appear as promising therapeutic targets for novel analgesic drugs [5]. It is also interesting to know whether mambalgins have other effects in brain besides pain modulation [9].

2.4. PhcrTx1

PhcrTx1 represents a newly discovered peptide, which was isolated from the sea anemones *Pseudacris crucifer* [64]. Structurally, it contains 32 amino acid residues. This peptide reveals an inhibitor cystine knot scaffold, which has been found in other venomous organisms, such as spider, scorpions and cone snails. Pharmacologically, PhcrTx1 inhibits peak ASIC currents in DRG neurons of rats with an IC_{50} of 0.1 μM. It does not affect the sustained component of the ASIC current or its desensitization rate. Furthermore, the toxin shows its effect in a closed state of the ASICs rather than an open state. PhcrTx1 also inhibits voltage-gated K^+, but not voltage-gated Na^+, currents in rat DRG neurons with an IC_{50} of 3.4 and 3.5 μM for peak and steady-state component, respectively. However, PhcrTx1 inhibits voltage-gated K^+ currents in DRG neurons, but with significantly lower potency and efficacy than its ability for inhibition on ASIC currents. Thus, PhcrTx1 represents the frontrunner of a novel structural group of sea anemone toxin that acts on both ASICs and Kv channels with high and low potency, respectively [64]. It is interesting to know whether PhcrTx1 plays any functional role in ASICs.

2.5. MitTx

In 2011, MitTx was discovered from the venom of the Texas coral snake *Micrurus tener tener* [6]. Structurally, peptide MixTx contains two subunits (MitTx-α and MitTx-β) with a β-bungarotoxin-like structure. The MitTx-α subunit has a 60 amino-acid Kunitz-type peptide and the MitTx-β subunit consists of a 120 amino-acid phospholipase A2-like protein. They can associate with each other in a 1:1 ratio (Kd: 12 nM), but this interaction is non-covalent, unlike the β-bungarotoxins that are linked by an interchain disulfide bond. Pharmacologically, MitTx, unlike other inhibitory toxins for ASICs, strongly activates several homomeric and heteromeric ASICs [6, 7]. MitTx produces long-lasting profound effects on homomeric rodent ASIC1a and ASIC1b currents (EC_{50}: 9 and 23 nM, respectively) and a much lower effect on ASIC3 current ($EC_{50:}$ 830 nM). During physiological pH condition (e.g. pH 7.4), MitTx reveals subtle effects on ASIC2a current, but potently enhances the ASIC current by shifting its activation curve toward less acidic pH. The effects of MitTx on sensory ganglion neurons from ASIC1a knockout mice were disappeared. Collectively, the data further suggest that effects of MixTx depend on ASIC1a-containing channels [6].

MitTx triggers a strong ASIC current in cultured sensory neurons in wild-type mice; these currents are lost in neurons from ASIC1a-knockout, but not from ASIC3-knockout mice. Consistent with this idea, injection of MitTx in the mice hindpaw displays a strong pain-related behavior (licking response). This effect is reduced in ASIC1a knockout mice but persists in ASIC3 knockout mice, suggesting the contribution of peripheral ASIC1a-containing channels in cutaneous pain [6]. It is needed to explore why MitTx produces lost-lasting effects in physiological concentration of pH on ASICs.

3. Conclusion

PcTx1 was the first peptide toxin found to block homomeric ASIC1a and heteromeric ASIC1a/2b channels. APETx2 was the second ASIC-targeting peptide discovered, and it inhibits ASIC3 channels. MitTx was discovered in 2011 and is a strong activator of ASICs during physiological conditions. Mambalgins have strong inhibition on ASIC1 channels. Another sea anemone peptide PhcrTx1 inhibits ASIC currents in DRG neurons. These peptide toxins have been very important to better understand the structure-function relationships of ASICs and their implication in physiological and pathological processes [5, 17]. ASIC-targeting peptides isolated from animal venoms that selectively block this class of channels are therefore not only instrumental as pharmacological tools to explore their function but also represent molecules of great potential therapeutic value [5]. ASIC channels appear therefore as targets for drug development in a variety of pathophysiological conditions [9].

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References

[1] Alvarez de la Rosa D, Krueger SR, Kolar A, Shao D, Fitzsimonds RM, Canessa CM. Distribution, subcellular localization and ontogeny of ASIC1 in the mammalian central nervous system. The Journal of Physiology. 2003;**546**:77-87. DOI: 10.1113/jphysiol.2002.030692

[2] Arias RL, Sung ML, Vasylyev D, Zhang MY, Albinson K, Kubek K, Kagan N, Beyer C, Lin Q, Dwye JM, Zaleska MM, Bowlby MR, Dunlop J, Monaghan M. Amiloride is neuroprotective in an MPTP model of Parkinson's disease. Neurobiology of Disease. 2008;**31**:334-341. DOI: 10.1016/j.nbd.2008.05.008

[3] Askwith CC, Wemmie JA, Price MP, Rokhlina T, Welsh MJ. Acid-sensing ion channel 2 (ASIC2) modulates ASIC1 H^+-activated currents in hippocampal neurons. The Journal of Biological Chemistry. 2004;**279**(18):18296-18305. DOI: 10.1074/jbc.M312145200

[4] Baron A, Diochot S, Salinas M, Deval E, Noël J, Lingueglia E. Venom toxins in the exploration of molecular, physiological and pathophysiological functions of acid-sensing ion channels. Toxicon. 2013;**75**:187-204. DOI: 10.1016/j.toxicon.2013.04.008

[5] Baron A, Lingueglia E. Pharmacology of acid-sensing ion channels—Physiological and therapeutical perspectives. Neuropharmacology. 2015;**94**:19-35. DOI: 10.1016/j.neuropharma. 2015.01.005

[6] Bohlen CJ, Chesler AT, Sharif-Naeini R, Medzihradszky KF, Zhou S, King D, Sanchez EE, Burlingame AL, Basbaum AI, Julius DA. Heteromeric Texas coral snake toxin targets acid-sensing ion channels to produce pain. Nature. 2011;**479**:410-414. DOI: 10.1038/nature10607

[7] Bohlen CJ, Julius D. Receptor-targeting mechanisms of pain causing toxins: How ow? Toxicon. 2012;**60**:254-264. DOI: 10.1016/j.toxicon.2012.04.336

[8] Chen CC, Zimmer A, Sun WH, Hall J, Brownstein MJ. A role for ASIC3 in the modulation of high-intensity pain stimuli. Proceedings of the National Academy of Sciences of the United States of America. 2002;**99**:8992-8997. DOI: 10.1073/pnas.122245999

[9] Chu XP, Grasing KA, Wang JQ. Acid-sensing ion channels contribute to neurotoxicity. Translational Stroke Research. 2014;**5**(1):69-78. DOI: 10.1007/s12975-013-0305-y

[10] Chu XP, Papasian CJ, Wang JQ, Xiong ZG. Modulation of acid-sensing ion channels: Molecular mechanisms and therapeutic potential. International Journal of Physiology, Pathophysiology and Pharmacology. 2011;**3**(4):288-309

[11] Chu XP, Wemmie JA, Wang WZ, Zhu XM, Saugstad JA, Price MP, Simon RP, Xiong ZG. Subunit-dependent high-affinity zinc inhibition of acid sensing ion channels. The Journal of Neuroscience. 2004;**24**(40):8678-8689. DOI: 10.1523/JNEUROSCI.2844-04.2004

[12] Chu XP, Xiong ZG. Physiological and pathological functions of acid-sensing ion channels in the central nervous system. Current Drug Targets. 2012;**13**(2):263-271

[13] Chung WS, Farley JM, Swenson A, Barnard JM, Hamilton G, Chiposi R, Drummond HA. Extracellular acidosis activates ASIC-like channels in freshly isolated cerebral artery smooth muscle cells. American Journal of Physiology-Cell Physiology. 2010;**298**:C1198-C1208. DOI: 10.1152/ajpcell.00511.2009

[14] Coryell MW, Wunsch AM, Haenfler JM, Allen JE, McBride JL, Davidson BL, Wemmie JA. Restoring acid-sensing ion channel-1a in the amygdala of knock-out mice rescues fear memory but not unconditioned fear responses. The Journal of Neuroscience. 2008;**28**:13738-13741. DOI: 10.1523/JNEUROSCI.3907-08.2008

[15] Coryell MW, Wunsch AM, Haenfler JM, Allen JE, Schnizler M, Ziemann AE, Cook MN, Dunning JP, Price MP, Rainier JD, Liu Z, Light AR, Langbehn DR, Wemmie JA. Acid-sensing ion channel-1a in the amygdala, a novel therapeutic target in depression related behavior. The Journal of Neuroscience. 2009;**29**:5381-5388. DOI: 10.1523/JNEUROSCI.0360-09.2009

[16] Coryell MW, Ziemann AE, Westmoreland PJ, Haenfler JM, Kurjakovic Z, Zha XM, Price M, Schnizler MK, Wemmie JA. Targeting ASIC1a reduces innate fear and alters neuronal activity in the fear circuit. Biological Psychiatry. 2007;**62**:1140-1148. DOI: 10.1016/j.biopsych.2007.05.008

[17] Cristofori-Armstrong B, Rash LD. Acid-sensing ion channel (ASIC) structure and function: Insights from spider, snake and sea anemone venoms. Neuropharmacology. 2017. pii: S0028-3908(17)30192-2. DOI: 10.1016/j.neuropharm.2017.04.042

[18] Deval E, Gasull X, Noel J, Salinas M, Baron A, Diochot S, Lingueglia E. Acid-sensing ion channels (ASICs): Pharmacology and implication in pain. Pharmacology & Therapeutics. 2010;**128**:549-558. DOI: 10.1016/j.pharmthera.2010.08.006

[19] Deval E, Noel J, Gasull X, Delaunay A, Alloui A, Friend V, Eschalier A, Lazdunski M, Lingueglia E. Acid-sensing ion channels in postoperative pain. The Journal of Neuroscience. 2011;**31**:6059-6066. DOI: 10.1523/JNEUROSCI.5266-10.2011

[20] Deval E, Noel J, Lay N, Alloui A, Diochot S, Friend V, Jodar M, Lazdunski M, Lingueglia E. ASIC3, a sensor of acidic and primary inflammatory pain. The EMBO Journal. 2008;**27**:3047-3055. DOI: 10.1038/emboj.2008.213

[21] Deval E, Salinas M, Baron A, Lingueglia E, Lazdunski M. ASIC2b-dependent regulation of ASIC3, an essential acid-sensing ion channel subunit in sensory neurons via the

partner protein PICK-1. The Journal of Biological Chemistry. 2004;**279**:19531-19539. DOI: 10.1074/jbc.M313078200

[22] Diochot S, Baron A, Rash LD, Deval E, Escoubas P, Scarzello S, Salinas M, Lazdunski MA. New sea anemone peptide, APETx2, inhibits ASIC3, a major acid-sensitive channel in sensory neurons. The EMBO Journal. 2004;**23**:1516-1525. DOI: 10.1038/sj.emboj.7600177

[23] Diochot S, Baron A, Salinas M, Douguet D, Scarzello S, Dabert-Gay AS, Debayle D, Friend V, Alloui A, Lazdunski M, Lingueglia E. Black mamba venom peptides target acid-sensing ion channels to abolish pain. Nature. 2012;**490**:552-555. DOI: 10.1038/nature11494

[24] Drew LJ, Rohrer DK, Price MP, Blaver KE, Cockayne DA, Cesare P, Wood JN. Acid-sensing ion channels ASIC2 and ASIC3 do not contribute to mechanically activated currents in mammalian sensory neurones. The Journal of Physiology. 2004;**556**:691-710. DOI: 10.1113/jphysiol.2003.058693

[25] Drummond HA, Jernigan NL, Grifoni SC. Sensing tension: Epithelial sodium channel/acid-sensing ion channel proteins in cardiovascular homeostasis. Hypertension. 2008;**51**:1265-1271. DOI: 10.1161/HYPERTENSIONAHA.107.093401

[26] Du J, Reznikov LR, Price MP, Zha XM, Lu Y, Moninger TO, Wemmie JA, Welsh MJ. Protons are a neurotransmitter that regulates synaptic plasticity in the lateral amygdala. Proceedings of the National Academy of Sciences of the United States of America. 2014;**111**:8961-8966. DOI: 10.1073/pnas.1407018111

[27] Dubé GR, Lehto SG, Breese NM, Baker SJ, Wang X, Matulenko MA, Honoré P, Stewart AO, Moreland RB, Brioni JD. Electrophysiological and in vivo characterization of A-317567, a novel blocker of acid sensing ion channels. Pain. 2005;**117**(1-2):88-96. DOI: 10.1016/j.pain.2005.05.021

[28] Dutertre S, Lewis RJ. Use of venom peptides to probe ion channel structure and function. The Journal of Biological Chemistry. 2010;**285**:13315-13320. DOI: 10.1074/jbc.R109.076596

[29] Dwyer JM, Rizzo SJ, Neal SJ, Lin Q, Jow F, Arias RL, Rosenzweig-Lipson S, Dunlop J, Beyer CE. Acid sensing ion channel (ASIC) inhibitors exhibit anxiolytic-like activity in preclinical pharmacological models. Psychopharmacology. 2009;**203**:41-52. DOI: 10.1007/s00213-008-1373-7

[30] Escoubas P, Bernard C, Lambeau G, Lazdunski M, Darbon H. Recombinant production and solution structure of PcTx1, the specific peptide inhibitor of ASIC1a proton-gated cation channels. Protein Science. 2003;**12**:1332-1343. DOI: 10.1110/ps.0307003

[31] Escoubas P, De Weille JR, Lecoq A, Diochot S, Waldmann R, Champigny G, Moinier D, Menez A, Lazdunski M. Isolation of a tarantula toxin specific for a class of proton-gated Na^+ channels. The Journal of Biological Chemistry. 2000;**275**:25116-25121. DOI: 10.1074/jbc.M003643200

[32] Feldman DH, Horiuchi M, Keachie K, McCauley E, Bannerman P, Itoh A, Itoh T, Pleasure D. Characterization of acid-sensing ion channel expression in oligodendrocyte-lineage cells. Glia. 2008;**56**:1238-1249. DOI: 10.1002/glia.20693

[33] Friese MA, Craner MJ, Etzensperger R, Vergo S, Wemmie JA, Welsh MJ, Vincen A, Fugge L. Acid-sensing ion channel-1 contributes to axonal degeneration in autoimmune inflammation of the central nervous system. Nature Medicine. 2007;**13**:1483-1489. DOI: 10.1038/nm1668

[34] Fromy B, Lingueglia E, Sigaudo-Roussel D, Saumet JL, Lazdunski M. Asic3 is a neuronal mechanosensor for pressure-induced vasodilation that protects against pressure ulcers. Nature Medicine. 2012;**18**:1205-1207. DOI: 10.1038/nm.2844

[35] Gründer S, Chen X. Structure, function, and pharmacology of acid sensing ion channels (ASICs): Focus on ASIC1a. International Journal of Physiology, Pathophysiology and Pharmacology. 2010;**2**:73-94

[36] Hayes SG, Kindig AE, Kaufman MP. Blockade of acid sensing ion channels attenuates the exercise pressor reflex in cats. The Journal of Physiology. 2007;**581**:1271-1282. DOI: 10.1113/jphysiol.2007.129197

[37] Hesselager M, Timmermann DB, Ahring PK. pH dependency and desensitization kinetics of heterologously expressed combinations of acid-sensing ion channel subunits. The Journal of Biological Chemistry. 2004;**279**(12):11006-11015. DOI: 10.1074/jbc.M313507200

[38] Housley DM, Housley GD, Liddell MJ, Jennings EA. Scorpion toxin peptide action at the ion channel subunit level. Neuropharmacology: pii: S0028-3908(16), 30454-3. 2016. DOI: 10.1016/j.neuropharm.2016.10.004

[39] Hu R, Duan B, Wang D, Yu Y, Li W, Luo H, Lu P, Lin J, Zhu G, Wan Q, Feng H. Role of acid-sensing ion channel 1a in the secondary damage of traumatic spinal cord injury. Annals of Surgery. 2011;**254**:353-362. DOI: 10.1097/SLA.0b013e31822645b4

[40] Huda R, Pollema-Mays SL, Chang Z, Alheid GF, McCrimmon D, Martina M. Acid-sensing ion channels contribute to chemosensitivity of breathing-related neurons of the nucleus of the solitary tract. The Journal of Physiology. 2012;**590**(19):4761-4775. DOI: 10.1113/jphysiol.2012.232470

[41] Ikeuchi M, Kolker SJ, Burnes LA, Walder RY, Sluka KA. Role of ASIC3 in the primary and secondary hyperalgesia produced by joint inflammation in mice. Pain. 2008;**137**:662-669. DOI: 10.1016/j.pain.2008.01.020

[42] Ikeuchi M, Kolker SJ, Sluka KA. Acid-sensing ion channel 3 expression in mouse knee joint afferents and effects of carrageenan induced arthritis. The Journal of Pain. 2009;**10**:336-342. DOI: 10.1016/j.jpain.2008.10.010

[43] Izumi M, Ikeuchi M, Ji Q, Tani T. Local ASIC3 modulates pain and disease progression in a rat model of osteoarthritis. Journal of Biomedical Science. 2012;**19**:77. DOI: 10.1186/1423-0127-19-77

[44] Jasti J, Furukawa H, Gonzales EB, Gouaux E. Structure of acid sensing ion channel 1 at 1.9A resolution and low pH. Nature. 2007;**449**:316-323. DOI: 10.1038/nature06163

[45] Jiang Q, Li MH, Papasian CJ, Branigan D, Xiong ZG, Wang JQ, Chu XP. Characterization of acid-sensing ion channels in medium spiny neurons of mouse striatum. Neuroscience. 2009;**162**(1):55-66. DOI: 10.1016/j.neuroscience.2009.04.029

[46] Jones NG, Slater R, Cadiou H, McNaughton P, McMahon SB. Acid-induced pain and its modulation in humans. Journal of Neuroscience. 2004, 2004;**24**:10974-10979. DOI: 10.1523/JNEUROSCI.2619-04.2004

[47] Karczewski J, Spencer RH, Garsky VM, Liang A, Leitl MD, Cato MJ, Cook SP, Kane S, Urban MO. Reversal of acid-induced and inflammatory pain by the selective ASIC3 inhibitor, APETx2. British Journal of Pharmacology. 2010;**161**:950-960. DOI: 10.1111/j.1476-5381.2010.00918.x

[48] Krishtal OA, Pidoplichko VI. A receptor for protons in the nerve cell membrane. Neuroscience. 1980;**5**(12):2325-2327. DOI: 10.1016/0306-4522(81)90105-6

[49] Krishtal OA, Pidoplichko VI. Receptor for protons in the membrane of sensory neurons. Brain Research. 1981;**214**(1):150-154. DOI: 10.1016/0006-8993(81)90446-7

[50] Li WG, Xu TL. ASIC3 channels in multimodal sensory perception. ACS Chemical Neuroscience. 2011;**2**:26-37. DOI: 10.1021/cn100094b

[51] Lingueglia E, de Weille JR, Bassilana F, Heurteaux C, Sakai H, Waldmann R, Lazdunski M. A modulatory subunit of acid-sensing ion channels in brain and dorsal root ganglion cells. The Journal of Biological Chemistry. 1997;**272**:29778-29783. DOI: 10.1074/jbc.272.47.29778

[52] Mamet J, Baron A, Lazdunski M, Voilley N. Proinflammatory mediators, stimulators of sensory neuron excitability via the expression of acid-sensing ion channels. Journal of Neuroscience. 2002;**22**:10662-10670

[53] Mamet J, Lazdunski M, Voilley N. How nerve growth factor drives physiological and inflammatory expressions of acid-sensing ion channel 3 in sensory neurons. The Journal of Biological Chemistry. 2003;**278**:48907-48913. DOI: 10.1074/jbc.M309468200

[54] Mazzuca M, Heurteaux C, Alloui A, Diochot S, Baron A, Voilley N, Blondeau N, Escoubas P, Gelot A, Cupo A, Zimmer A, Zimmer AM, Eschalier A, Lazdunski M. A tarantula peptide against pain via ASIC1a channels and opioid mechanisms. Nature Neuroscience. 2007;**10**:943-945. DOI: 10.1038/nn1940

[55] McCord JL, Tsuchimochi H, Kaufman MP. Acid-sensing ion channels contribute to the metaboreceptor component of the exercise pressor reflex. American Journal of Physiology. Heart and Circulatory Physiology. 2009;**297**:H443-H449. DOI: 10.1152/ajpheart.00328.2009

[56] McMahon SB, Jones NG. Plasticity of pain signaling: Role of neurotrophic factors exemplified by acid-induced pain. Journal of Neurobiology. 2004;**61**:72-87. DOI: 10.1523/JNEUROSCI.2619-04.2004

[57] Miljanich GP. Ziconotide: Neuronal calcium channel blocker for treating severe chronic pain. Current Medicinal Chemistry. 2004;**11**:3029-3040. DOI: 10.2174/0929867043363884

[58] Molliver DC, Immke DC, Fierro L, Pare M, Rice FL, EW MC. ASIC3, an acid-sensing ion channel, is expressed in metaboreceptive sensory neurons. Molecular Pain. 2005;**1**:35. DOI: 10.1186/1744-8069-1-35

[59] Noël J, Salinas M, Baron A, Diochot S, Deval E, Lingueglia E. Current perspectives on acid-sensing ion channels: New advances and therapeutic implications. Expert Review of Clinical Pharmacology. 2010;**3**:331-346. DOI: 10.1586/ecp.10.13

[60] Pignataro G, Simon RP, Xiong ZG. Prolonged activation of ASIC1a and the time window for neuroprotection in cerebral ischaemia. Brain. 2007;**130**:151-158. DOI: 10.1093/brain/awl325

[61] Prashanth JR, Hasaballah N, Vetter I. Pharmacological screening technologies for venom peptide discovery. Neuropharmacology S0028-3908 (17), 30130-2. 2017. DOI: 10.1016/j.neuropharm.2017.03.038

[62] Price MP, Gong H, Parsons MG, Kundert JR, Reznikov LR, Bernardinelli L, Chaloner K, Buchanan GF, Wemmie JA, Richerson GB, Cassell MD, Welsh MJ. Localization and behaviors in null mice suggest that ASIC1 and ASIC2 modulate responses to aversive stimuli. Genes, Brain, and Behavior. 2014;**13**(2):179-194. DOI: 10.1111/gbb.12108

[63] Price MP, McIlwrath SL, Xie J, Cheng C, Qiao J, Tarr DE, Sluka KA, Brennan TJ, Lewin GR, Welsh MJ. The DRASIC cation channel contributes to the detection of cutaneous touch and acid stimuli in mice. Neuron. 2001;**32**:1071-1083. DOI: 10.1016/S0896-6273(01)00547-5

[64] Rodríguez AA, Salceda E, Garateix AG, Zaharenko AJ, Peigneur S, López O, Pons T, Richardson M, Díaz M, Hernández Y, Ständker L, Tytgat J, Soto E. A novel sea anemone peptide that inhibits acid-sensing ion channels. Peptides. 2014;**53**:3-12. DOI: 10.1016/j.peptides.2013.06.003

[65] Sarfo-Poku C, Eshun O, Lee KH. Medical application of scorpion venom to breast cancer: A mini-review. Toxicon. 2016;**122**:109-112. DOI: 10.1016/j.toxicon.2016.09.005

[66] Schmidtko A, Lotsch J, Freynhagen R, Geisslinger G. Ziconotide for treatment of severe chronic pain. Lancet. 2010;**375**:1569-1577. DOI: 10.1016/S0140-6736(10)60354-6

[67] Sherwood TW, Lee KG, Gormley MG, Askwith CC. Heteromeric acid-sensing ion channels (ASICs) composed of ASIC2b and ASIC1a display novel channel properties and contribute to acidosis-induced neuronal death. The Journal of Neuroscience. 2011;**31**(26):9723-9734. DOI: 10.1523/JNEUROSCI.1665-11.2011

[68] Sluka KA, Price MP, Breese NM, Stucky CL, Wemmie JA, Welsh MJ. Chronic hyperalgesia induced by repeated acid injections in muscle is abolished by the loss of ASIC3, but not ASIC1. Pain. 2003;**106**:229-239

[69] Sluka KA, Radhakrishnan R, Benson CJ, Eshcol JO, Price MP, Babinski K, Audette KM, Yeomans DC, Wilson SP. ASIC3 in muscle mediates mechanical, but not heat, hyperalgesia

associated with muscle inflammation. Pain. 2007;**129**:102-112. DOI: 10.1186/1744-8069-1-35

[70] Sluka KA, Winter OC, Wemmie JA. Acid-sensing ion channels: A new target for pain and CNS diseases. Current Opinion in Drug Discovery & Development. 2009;**12**:693-704

[71] Song N, Guan R, Jiang Q, Hassanzadeh CJ, Chu Y, Zhao X, Wang X, Yang D, Du Q, Chu XP, Shen L. Acid-sensing ion channels are expressed in the ventrolateral medulla and contribute to central chemoreception. Scientific Reports. 2016;**6**:38777. DOI: 10.1038/srep38777

[72] Song N, Zhang G, Geng W, Liu Z, Jin W, Li L, Cao Y, Zhu D, Yu J, Shen L. Acid sensing ion channel 1 in lateral hypothalamus contributes to breathing control. PloS One. 2012;**7**:e39982. DOI: 10.1371/journal.pone.0039982

[73] Steen KH, Issberner U, Reeh PW. Pain due to experimental acidosis in human skin: Evidence for non-adapting nociceptor excitation. Neuroscience Letters. 1995;**199**:29-32. DOI: 10.1016/0304-3940(95)12002-L

[74] Tan J, Ye X, Xu Y, Wang H, Sheng M, Wang F. Acid-sensing ion channel 1a is involved in retinal ganglion cell death induced by hypoxia. Molecular Vision. 2011;**17**:3300-3308

[75] Tsuchimochi H, Yamauchi K, McCord JL, Kaufman MP. Blockade of acid sensing ion channels attenuates the augmented exercise pressor reflex in rats with chronic femoral artery occlusion. The Journal of Physiology. 2011;**589**:6173-6189. DOI: 10.1113/jphysiol.2011.217851

[76] Ugawa S, Ueda T, Ishida Y, Nishigaki M, Shibata Y, Shimada S. Amiloride-blockable acid-sensing ion channels are leading acid sensors expressed in human nociceptors. The Journal of Clinical Investigation. 2002;**110**:1185-1190. DOI: 10.1172/JCI15709

[77] Voilley N, de Weille J, Mamet J, Lazdunski M. Nonsteroid anti-inflammatory drugs inhibit both the activity and the inflammation induced expression of acid-sensing ion channels in nociceptors. The Journal of Neuroscience. 2001;**21**:8026-8033

[78] Walder RY, Gautam M, Wilson SP, Benson CJ, Sluka KA. Selective targeting of ASIC3 using artificial miRNAs inhibits primary and secondary hyperalgesia after muscle inflammation. Pain. 2011;**152**:2348-2356. DOI: 10.1016/j.pain.2011.06.027

[79] Walder RY, Rasmussen LA, Rainier JD, Light AR, Wemmie JA, Sluka KA. ASIC1 and ASIC3 play different roles in the development of hyperalgesia after inflammatory muscle injury. The Journal of Pain. 2010;**11**:210-218. DOI: 10.1016/j.jpain.2009.07.004

[80] Waldmann R, Champigny G, Bassilana F, Heurteaux C, Lazdunski M. A proton-gated cation channel involved in acid-sensing. Nature. 1997;**386**(6621):173-177. DOI: 10.1038/386173a0

[81] Wang YZ, Xu TL. Acidosis acid-sensing ion channels, and neuronal cell death. Molecular Neurobiology. 2011;**44**:350-358. DOI: 10.1007/s12035-011-8204-2

[82] Wemmie JA, Askwith CC, Lamani E, Cassell MD, Freeman JH Jr, Welsh MJ. Acid-sensing ion channel 1 is localized in brain regions with high synaptic density and contributes to fear conditioning. Journal of Neuroscience. 2003;**23**:5496-5502

[83] Wemmie JA, Chen J, Askwith CC, Hruska-Hageman AM, Price MP, Nolan BC, Yoder PG, Lamani E, Hoshi T, Freeman JH Jr, Welsh MJ. The acid-activated ion channel ASIC contributes to synaptic plasticity, learning, and memory. Neuron. 2002;**34**:463-477. DOI: 10.1016/S0896-6273(02)00661-X

[84] Wemmie JA, Price MP, Welsh MJ. Acid-sensing ion channels: Advances, questions and therapeutic opportunities. Trends in Neurosciences. 2006;**29**(10):578-586. DOI: 10.1016/j.tins.2006.06.014

[85] Wemmie JA, Coryell MW, Askwith CC, Lamani E, Leonard AS, Sigmund CD, Welsh MJ. Overexpression of acid-sensing ion channel 1a in transgenic mice increases acquired fear-related behavior. Proceedings of the National Academy of Sciences of the United States of America. 2004;**101**:3621-3626. DOI: 10.1073/pnas.0308753101

[86] WL W, Cheng CF, Sun WH, Wong CW, Chen CC. Targeting ASIC3 for pain, anxiety, and insulin resistance. Pharmacology & Therapeutics. 2012;**134**:127-138. DOI: 10.1016/j.pharmathera. 2011.12.009

[87] Xiong ZG, Chu XP, Simon RP. Acid sensing ion channels–Novel therapeutic targets for ischemic brain injury. Frontiers in Bioscience. 2007;**12**:1376-1386

[88] Xiong ZG, Pignataro G, Li M, Chang SY, Simon RP. Acid sensing ion channels (ASICs) as pharmacological targets for neurodegenerative diseases. Current Opinion in Pharmacology. 2008;**8**:25-32. DOI: 10.1016/j.coph.2007.09.001

[89] Xiong ZG, Zhu XM, Chu XP, Minami M, Hey J, Wei WL, MacDonald JF, Wemmie JA, Price MP, Welsh MJ, Simon RP. Neuroprotection in ischemia: Blocking calcium-permeable acid-sensing ion channels. Cell. 2004;**118**:687-698. DOI: 10.1016/j.cell.2004.08.026

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Toxicologists seek to better understand the myriad of mechanisms responsible for neurotoxins. Because the incidence of neurobehavioral hazards and risks of exogenous compounds (e.g., natural toxins, synthetic molecules, and therapeutic agents) remain a subject matter of interest, predictive tools have evolved, including but not limited to novel translational in vitro models, biomarkers, newer epidemiological research tools, and well-accepted best practices for diagnosing neurotoxins in clinical practice. Taken together, the foreseen need to highlight some of the more appreciated and/or emerging tactical approaches in neurotoxicology results in a "one-stop reference" book, *Neurotoxins*.

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