toxins

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Review

Cyanotoxins and the Nervous System

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Abstract: Cyanobacteria are capable of producing a wide range of bioactive compounds with many considered to be toxins. Although there are a number of toxicological outcomes with respect to cyanobacterial exposure, this review aims to examine those which affect the central nervous system (CNS) or have neurotoxicological properties. Such exposures can be acute or chronic, and we detail issues concerning CNS entry, detection and remediation. Exposure can occur through a variety of media but, increasingly, exposure through air via inhalation may have greater significance and requires further investigation. Even though cyanobacterial toxins have traditionally been classified based on their primary mode of toxicity, increasing evidence suggests that some also possess neurotoxic properties and include known cyanotoxins and unknown compounds. Furthermore, chronic long-term exposure to these compounds is increasingly being identified as adversely affecting human health.

Keywords: cyanobacteria; toxin; CNS; chronic; acute; neurodegeneration

Key Contribution: This review summarizes what is known concerning cyanobacterial toxins that have neurotoxic potential, including toxins that have other primary toxicological modes of action.

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1. Introduction

Cyanobacteria are evolutionarily ancient photosynthetic organisms with fossils dating back 3.6 billion years [1]. Although previously referred to as blue-green algae, cyanobacteria are Gram-negative bacteria and have characteristics of such bacteria including the presence of lipopolysaccharide (LPS) in their cell wall [2]. They are common components of aquatic and terrestrial environments, and their growth is influenced by physical and chemical factors. In aquatic environments, conditions such as calm, sunny weather along with the provision of nutrients including nitrogen and phosphorous can result in mass accumulations of cyanobacteria, manifested as blooms and scums [3]. Often unsightly, and potentially containing compounds such as geosmin and methylisoborneol which give the water an unpleasant taste and odor, cyanobacteria can negatively affect the aesthetics of water [4]. As some cyanobacterial cells are capable of producing gas vesicles to provide buoyancy, this allows them to float to the surface resulting in the potential for exponential concentration factor increases in cell number. Consequently, under these conditions of positive buoyancy and the action of wind, scums resembling thick green paint can form on shorelines and in embayments [3]. Given that cyanobacteria are capable of producing a wide range of bioactive compounds, with many considered to be toxins (cyanotoxins), any toxins naturally present within these cyanobacterial cells are able to reach a concentration that may pose a risk to people and animals.

Although there is evidence that cyanobacteria have had the capacity to produce toxins for millions of years [5,6], the toxic effects of exposure to cyanobacteria have been known since at least the late 19th century, including reports and investigations into the toxicity of *Nodularia* scum in Australia [7]. Periodic mass mortalities of animals including

dogs, birds and cows have occurred with exposure to cyanobacteria which are often considered to be the proximal cause, given the detectable presence of cyanotoxins in clinical materials (e.g., [8–16]). From such observations, detailed investigations into toxic compounds produced by cyanobacteria have resulted in the elucidation of a number of compounds of human and animal health concern. Through rigorous assessments of axenic and/or monocyanobacterial laboratory cultures, environmental collections and clinical materials from animal and human intoxications, new and emerging toxic compounds produced by cyanobacteria continue to be characterized.

Although animals account for the majority of reported cyanobacterial intoxications, human populations exposed to cyanobacterial toxins have also resulted in reported illness and death. Examples include: outbreaks of gastroenteritis from exposure to LPS [17,18], hepatomegaly from (presumed) exposure to cylindrospermopsin [19] and pneumonia-like symptoms in army cadets after oral exposure to *Microcystis* scum during aquatic drill exercises [20]. These and other cases highlight the potential risk of acute exposure to cyanobacterial toxins. The most high-profile case of human exposure to cyanobacterial toxins occurred in Caruaru, Brazil in 1996. At a haemodialysis clinic, 100 people died and 52 people had confirmed exposure to the hepatotoxic microcystins through intravenous administration of water ineffectively treated at the clinic and obtained from a lake known to harbor cyanobacterial blooms [21,22]. Later assessment and analysis of materials related to these intoxications determined that the cytotoxic cylindrospermopsins were also present [23]. During this poisoning event, many of the patients exposed to this ineffectively treated water complained of neurological symptoms such as tinnitus, dizziness, vertigo and vision issues [21,22]. This led to the idea that cyanotoxins not traditionally classified as being neurotoxic may demonstrate neurological effects and may negatively interact with the mammalian central nervous system (CNS). Therefore, it may be necessary to expand the known modes of action of cyanobacterial toxins to take into account other less-reported toxic effects. Consequently, this review aims to examine what is currently known about cyanotoxins that can affect the nervous system, particularly the CNS.

2. Toxins That Affect the Brain and Nerves

Traditionally, cyanotoxins have been categorized with respect to what major aspects of mammalian physiology are adversely affected (e.g., cytotoxins, hepatotoxins and gastrointestinal toxins). Cyanobacterial neurotoxins (Figure 1) are an additional class of compounds with demonstrated neurological effects as the principal known mode of action [4]. However, other toxic cyanobacterial compounds not traditionally considered neurotoxins have been increasingly found to have neurological effects or are able to enter the CNS (e.g., [24]). Therefore, for the purposes of this review, cyanotoxins with neurotoxic effects have been divided into the following categories:

2.1. Traditional Acute Neurotoxins

These are largely alkaloid or organosphosphorous compounds that are extremely potent in mammalian systems. Their effects are largely observed within minutes to hours. Although chronic, long-term damage cannot be excluded, if the victim survives initial intoxication (either naturally or through medical intervention), no long-lasting adverse health effects are currently known to occur.

2.1.1. Anatoxin-a and Homologues

This class of low molecular weight alkaloid toxins encompasses about 7 cyanobacterially-derived compounds and degradation products, which can be expanded through known synthetic analogs [25,26]. The two anatoxin-a variants produced by cyanobacteria are anatoxin-a and homoanatoxin-a [26]. They are fast acting, function as acetylcholine mimics that bind to nicotinic acetylcholine receptors and result in the inability of acetylcholine esterase to remove these toxins from this essential CNS receptor [27]. Subsequently, this results in continued excitation and depolarization of neurons and can cause paralysis, asphyx-

iation and death when exposed to a sufficiently high concentration. In the CNS anatoxin-a has been shown to affect dopamine [28], blood pressure and heart rate [29]. Furthermore, anatoxin-a has been attributed to an outbreak of acute human poisoning resembling a "cerebellar syndrome" (including ataxia, dizziness and visual disturbances) following consumption of tunicates in southern France [30,31]. To a lesser extent, anatoxin-a can affect muscarinic acetylcholine receptors [27]. Often produced by filamentous cyanobacteria such as *Phormidium* in benthic mats, when associated with the co-production of taste and odour compounds, anatoxin-a has resulted in the deaths of dogs who consume these mats, either from waterbodies or from filaments that adhere to the fur of the animals [14]. When birds are affected by anatoxin-a, one observed physiological response is opisthotonous, whereby the muscle at the back of the neck is affected and contracts, resulting in the head lying along the back of the bird [26,32].

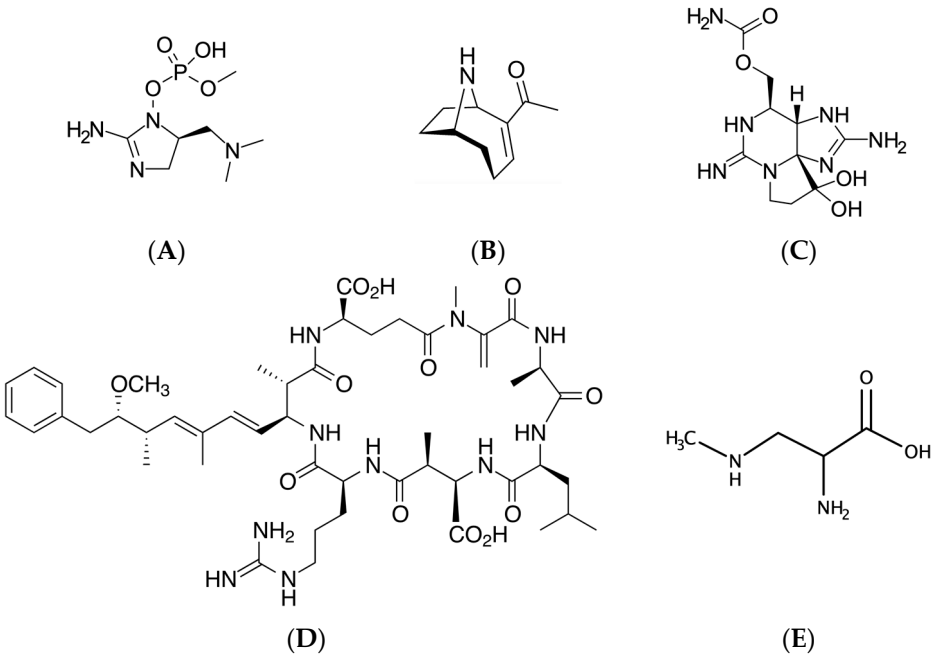

Figure 1. Structures of cyanotoxins with potential neurotoxicological implications. (**A**), guanitoxin; (**B**), anatoxin-a; (**C**), saxitoxin; (**D**), microcystin-LR; (**E**), β-N-methylamino-L-alanine.

2.1.2. Saxitoxins

Traditionally considered a product of marine dinoflagellates, saxitoxins are a group of 57 guanidinium alkaloids that are responsible for paralytic shellfish toxin poisoning in marine environments, largely through the consumption of contaminated shellfish [33,34]. The saxitoxins themselves are further divided, dependent on their chemical structures and substitutions, into classes such as C toxins, G toxins and LW toxins [33,35]. Of the acute cyanobacterial neurotoxins, saxitoxins have been known to cause human deaths as a result of their production by marine dinoflagellates which then contaminate shellfish with this highly potent neurotoxin [36]. Saxitoxins can be produced by cyanobacterial genera including *Aphanizomenon*, *Dolichospermum* and *Cylindrospermopsis* and blooms of these organisms have the potential to be highly toxic to animals and humans [4]. The majority of saxitoxin variants produced by cyanobacteria include saxitoxin and neosaxitoxin, in addition to LW (*Lyngbya wollei*) toxins [35]. Saxitoxins act by blocking voltage-gated sodium channels [37] and if present in sufficiently high concentration can result in paralysis and death. Other neuronal channels such as potassium and calcium channels may also be affected by saxitoxins [38].

2.1.3. Anatoxin-a(S) (Guanitoxin)

A naturally occurring organophosphate, anatoxin-a(*S*) has been associated with *Anabaena* (*Dolichospermum*) blooms, particularly in Danish lakes where it has resulted in the deaths of waterfowl [12]. Now renamed guanitoxin [39] and similar in structure to organophosphate pesticides and insecticides, this toxin is able to inhibit acetylcholine esterase [39–42], an essential enzyme that removes acetylcholine from the synapse in mammalian neurons. Consequently, inactivation of this enzyme by guanitoxin can result in paralysis and asphyxiation and is also used in vitro as a diagnostic test for its presence [40]. Using such an assay, its presence has been inferred in desert crust assemblages of cyanobacteria largely comprised of the genus *Microcoleus* [43]. Although not common in terrestrial environments, intoxications of dogs that drink water overlying these desert crusts have been observed [16]. Although originally similar in name to anatoxin-a, guanitoxin is dramatically different to anatoxin-a and, as with other organophosphates, poisoning results in hypersalivation, as denoted by *S* in the original name for guanitoxin [39,42].

2.2. Neurotoxins Associated with Neurodegeneration

As described above, acute exposure to certain cyanobacteria and their toxins is relatively well known. However, there is also on-going research investigating the long-term effects and/or chronic exposure to cyanobacterial toxins. For example, there is increasing evidence that microcystins, nodularin and cylindrospermopsin all have potential deleterious effects with respect to tumor promotion and cancer after exposure to concentrations that in the short term are unlikely to result in illness (e.g., [44–47]). Many parts of the human body are able to deal with toxicants through dilution (cell division), detoxication and metabolism. However, the CNS contains neurons, which are large, non-dividing cells with substantial metabolic demands and may accumulate damage with age [48] and encountered insults. Consequently, this population of highly specialized cells may be particularly susceptible to the long-term actions of low concentrations of toxic compounds. To date, there are several cyanobacterial toxins that have been associated with neurodegeneration.

2.2.1. BMAA and Isomers

Increasingly, neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS; also known as motor neurone disease) are thought to have an environmental component and do not arise as a consequence of genetics alone [49]. Thus, studying compounds associated with and identified as risk factors for these diseases may lead to clues concerning disease etiology and treatments. One particular disease and case study is ALS/Parkinsonism dementia complex (ALS/PDC), which was at one time known to be prevalent on the island of Guam [50]. Analysis of various factors associated with ALS/PDC identified diet as an important correlate in terms of developing this disease [51]. Further analyses of indigenous diets found that certain popular foodstuffs were high in a novel amino acid called β-*N*-methylamino-L-alanine (BMAA) that, when fed to chicks, resulted in neurotoxicity [52,53]. Current research suggests that BMAA can be produced by cyanobacteria [54,55], marine [56] and freshwater [57] diatoms and some chemoheterotrophic bacteria, such as members of the genus *Paenibacillus* spp. [58]. Increasingly the link between BMAA detection and the occurrence of dinoflagellates in marine waters suggests that this group of potentially harmful photosynthetic algae may also be capable of producing BMAA [59,60].

Although Guam was a focus for BMAA and ALS/PDC, the examination of Guamanian and non-Guamanian human brains showed that BMAA was present in other geographic locations [61,62]. Moreover, the work of Pablo and colleagues also showed that this amino acid is associated with additional neurodegenerative diseases such as ALS and Alzheimer's disease (AD) [62]. Intriguingly, experiments feeding non-human primates with BMAA showed the development of hallmarks of human neurodegenerative diseases such as β-amyloid plaques and neurofibrillary tangles (hallmarks of AD as well as Guamanian ALS/PDC) as well as other neuropathological features (e.g., microglial activation) that are consistent with diseases such as ALS and AD [63,64]. While it is currently difficult

to definitively determine how significant BMAA exposure is as a causal factor in ALS or AD, BMAA has been increasingly implicated as a risk factor for human neurodegenerative disease and requires greater attention [65,66].

Including BMAA, four naturally occurring isomers are currently considered to exist. Studies using the isomers *N*-(2-aminoethyl)glycine (AEG) and 2,4-diaminobutyric acid (DAB) have shown these compounds to possess neurotoxicity. Within the CNS, AEG may potentially act upon different receptors than BMAA and, in some cases, be considered more toxic [67,68]. Although BMAA exposure may occur throughout the world, e.g., through the consumption of contaminated shellfish [69], further research is needed to understand the occurrence and toxicity of all these neurotoxic isomers as well as more fully determine the risk of exposure and their adverse effects on neurological health. Moreover, as these neurotoxic amino acids can co-occur in harmful algal and cyanobacterial blooms, such exposure scenarios raise additional concerns regarding the synergistic effects of cyanotoxins [70].

2.2.2. Aetokthonotoxin

The interaction of humans and animals with the environment has the potential for new and emerging diseases and toxicoses to be observed and reported. One example is the identification of avian vacuolar myelinopathy (AVM) in the brains of birds, particularly eagles that feed near lakes [71,72]. Certain lakes are known to be sites where outbreaks of this disease occur and, until recently, no known toxicants have been definitively identified. The disease was associated with exposure to *Hydrilla*, and epiphytic cyanobacteria present on the surface of this plant were considered as potential causative agents [73]. Feeding test animals extracts of certain collections of *Hydrilla* resulted in gross pathology consistent with AVM, and epiphytic cyanobacteria were also shown to be toxic to bird species such as coots that were fed these extracts [74]. Extensive research has now identified a brominated cyanobacterial toxin, aetokthonotoxin, which results in the production of vacuoles and lesions in the brains of birds fed this toxin, consistent with it being a cause of AVM [74].

2.3. Cyanotoxins with Potential Neurological Effects

2.3.1. Microcystins

Microcystins are common cyclic peptides that are known to be produced by cyanobacterial genera such as *Microcystis*, *Anabaena* and *Planktothrix* [4]. They are comprised of 7 amino acids and due to substitutions and modifications of the structure, over 240 variants are currently known, each with differing toxicities [75,76]. Exposure to microcystins has resulted in the deaths of animals such as cows and dogs and are one of only a few cyanotoxins that have regulations for permissible concentrations in drinking and recreational waters [8]. Although traditionally classified as a hepatotoxins, people who were exposed to microcystins complained of neurological symptoms such as tinnitus and vision problems, leading to the idea that microcystins may possess adverse neurological activities [21–24].

Through investigations examining the hepatotoxic effects of microcystins, one molecular mode of action is the inhibition of protein phosphatases and phosphoprotein phosphatases [77,78]. Protein phosphatases are key cellular enzymes that are also found within the CNS and their inhibition has been implicated in the development of neurodegenerative diseases [79]. Although the permissible concentrations of microcystins in drinking and recreational waters include the potential for tumor promotion, current and future determinations of permissible microcystin concentrations are considering their potential for neurotoxicity [80].

2.3.2. Lipopeptides

Assessment of *Lyngbya majuscula* (*Moorea producens*) has shown that this marine cyanobacterium is capable of producing a wide range of bioactive compounds with unusual and unique toxicological actions [81]. A number of lipopeptides that contain an aliphatic group can be produced by cyanobacteria and are capable of affecting voltage

gated sodium channels on neurons. Using N-methyl-D-aspartate (NMDA) agonists, the action of antillatoxins and kalkitoxin on cerebellar granule cells could be prevented [82]. Further differences have been observed between the lipopetides with antillatoxins having the potential to activate voltage gated sodium channels, whereas jamaicamides and kalkitoxin inhibit these channels, in a manner similar to saxitoxin [25,83,84]. The assessment of *L. majuscula* shows the importance of assessing cyanobacteria from a wide range of environments for compounds with toxic and therapeutic potential as such voltage-gated sodium channel inhibitors may be applicable as analgesics [85,86].

2.3.3. Cylindrospermopsin

Although traditionally considered a cytotoxin, cylindrospermopsin was identified following an assessment of cyanobacterial bloom material from a human poisoning incident on Palm Island, Australia [87,88]. Here, an outbreak of hepatic gastroenteritis resulted in the hospitalization of 140 people, with some showing an enlarged liver (hepatomegaly) [87]. Several studies have assessed the neurotoxic potential of this cyanotoxin and some of the responses seen could be a consequence of the general cytotoxic action of cylindrospermopsin, e.g. affecting reactive oxygen species and DNA damage (reviewed by [89]). Using extracts and purified fractions from *Cylindrospermopsis raciborskii*, Kiss et al. [90] showed a neuroactive effect of these preparations on snail neurons, although Vehovsvky et al. [91] considered that this effect was due to an anatoxin-a-like compound. Using cell lines, Tasker et al. [92] showed cylindrospermopsin-induced apoptosis and inflammation in murine neuroblastoma and glial cells.

2.4. Other Cyanobacterial Compounds of Possible Neurotoxicological Interest

In addition to the known cyanobacterial toxins, these photosynthetic Gram-negative organisms are capable of producing a plethora of bioactive substances [81]. Many of them are capable of inhibiting a variety of eukaryotic enzymes such as elastase, trypsin and chymotrypsin (reviewed by [81]). Of potential interest to neurotoxicity are compounds such as Anabaenopeptin F, Oscillamides B and C and cyanostatins A and B, which have the potential to inhibit protein phosphatases [93,94], enzymes implicated in the development of neurodegenerative disease [79] and one of the proposed mechanisms of microcystin neurotoxicity [24]. Other aspects of neurology that may be influenced by cyanobacterial compounds include Anabaenopeptin F and biogenic amines that may influence or mimic the effect of norepinephrine [81]. Nodularin is potentially a compound of interest and has been associated with both human and animal poisonings. Although there is limited neurotoxic evidence, nodularin is structurally and toxicologically similar to microcystin, a cyanotoxin with likely neurotoxic properties [24]. A component of the outer membrane of Gram-negative bacteria, LPS is a known gastro-intestinal irritant which has been implicated as the causative agent of a number of human poisoning incidents [2]. Furthermore, as it is a component of the outer membrane of Gram-negative bacteria then chemoheterotrophic bacteria are also capable of producing this complex. Although compositions of LPS can differ between chemoheterotrophic bacteria and cyanobacteria [2,95] and it is not a known neurotoxin, some reports have shown that *E. coli* LPS can induce the formation of lipocalin-2, affecting the expression of chemokines in the CNS which may then affect cell migration and inflammation [96].

3. Transportation into the CNS

When the blood–brain barrier (BBB) is intact, then transporters may be required to allow compounds, such as cyanotoxins, to enter the CNS. However, if the BBB is damaged, then this would likely increase the range of compounds that could enter the CNS and cause adverse effects [97]. Preventing cyanotoxins from entering the CNS may serve as a therapeutic target during intoxications. Although this strategy may have little effect on acute intoxications, where high toxin concentrations rapidly affect the CNS, such research

may benefit those who are chronically exposed to cyanotoxins as a means to prevent adverse, long-term health effects.

In addition to identifying transporters for individual cyanotoxins, likely CNS transporters might be inferred from the examination of structurally and toxicologically related compounds. Anatoxin-a has been shown to be synthesized from cocaine [27], an alkaloid that displays neurological effects and, with long-term use, is associated with cognitive decline [98]. Although anatoxin-a has been shown to cause a release of dopamine, rather than affecting dopamine and serotonin receptors such as with cocaine, anatoxin-a is an acetylcholine mimic [99,100]. The most common acetylcholine mimic people are exposed to is nicotine and anatoxin-a affects nicotinic and to some extent muscarinic acetylcholine receptors [101]. Nicotine is able to enter the CNS extremely quickly, as evidenced by inhalation of tobacco combustion products. It is most likely transported by cation/organic molecule antiporter systems, which can be prevented by drugs such as verapamil and clonidine [102]. Although anatoxin-a can affect muscarinic acetylcholine receptors, muscarine is unable to cross the BBB and these receptors are found in many mammalian organs [103].

Guanitoxin is the only known naturally-occurring organophosphate molecule and is occasionally found in cyanobacterial blooms and potentially terrestrial assemblages of cyanobacteria [12,43]. Synthetic organophosphates have been widely used as pesticides and insecticides for many years and have been linked to neurodegenerative diseases such as Parkinson's disease (PD) and ALS. A wide range of such compounds are known and used including malathion, parathion and chlorpyrifos. They are effective as they inhibit acetylcholine esterase, an essential enzyme that removes acetylcholine from synapses [104]. Although unknown for exposure to guanitoxin, long-term exposure to some organophosphates has been linked to the development of cancer in farmers including bladder, prostate, kidney and lung cancer [105]. Given that organophosphates have been linked to both PD [106–108] and ALS [109,110], it is plausible that guanitoxin may exhibit chronic effects at relatively low concentrations. Moreover, certain organophosphates can induce a form of neurotoxicity, known as organophosphate-induced delayed polyneuropathy (OPIDP) [111]. There have been several occurrences of human poisonings resulting in OPIDP, mostly involving tri-*ortho*-cresyl phosphate, which functions as a weak AChE inhibitor [111]. Whether guanitoxin could lead to OPIDP is not known, but the mechanistic parallel and potential for human harm warrants investigation. Certain organophosphates such as soman, sarin, chlopyrifos and malathion are known to break down the BBB [112–114], thereby allowing entry of these and/or other compounds into the CNS where they are then capable of disrupting neuronal processes.

Saxitoxins are extremely potent inhibitors of voltage-gated sodium channels [33]. They are capable of crossing the BBB [115,116] and increase serotonin concentrations in rats at high doses [117]. However, the concentration to which an organism is exposed may determine whether there is sufficient saxitoxin biologically available to cross the BBB, as a result of its rapid binding to voltage-gated sodium channels throughout the body. Tetrodotoxin, another potent toxin that acts on voltage-gated sodium channels may provide insight as to the pharmacological action of saxitoxins, although studies so far indicate that tetrodotoxin is unable to cross the BBB [118]. Research suggests that long-term exposure to saxitoxins may cause adverse health effects on antioxidant systems and DNA, as demonstrated in fish and mammalian models [34].

Although microcystins are primarily considered to be hepatotoxins and tumor promoters, their potential neurotoxicity is receiving increasing attention. In terms of CNS transport, multi-organ organic anion transport system (OATP) proteins have been implicated as allowing compounds to cross the BBB and, of this large family of transport proteins, human OATP1A2 may transport microcystins [119–121]. Although further research is required to understand whether this happens in the human brain, Furstein et al. [121] showed that differences in transport can be observed, where microcystin-LF may be more efficiently transported than the more hydrophobic, microcystin-LR. This observation is in

keeping with the potential differences in toxicity between microcystin variants and their hydrophobicities [122].

Cylindrospermopsins are another class of cyanobacterial toxins that may also have neurological implications [89], with inflammatory effects observed in microglial and neuroblast cell lines [92] and neurotoxicity observed in tilapia [123]. Although there are no known transporters for cylindrospermopsins across the BBB or evidence of passive diffusion [124], enzyme-linked immunosorbent assay (ELISA) analysis of fish brains has shown the presence of this neurotoxin. However, as false positives can be observed with commercial cylindrospermopsin ELISAs [125], further research on the transportation and presence of this cyanotoxin in the brain are required.

The neurotoxic amino acid, BMAA, is known to accumulate in the brain after exposure [126] and has been found in human and dolphin brains [62,127]. Experiments concerning potential transporters identified the large neutral amino acid transporter [128], as this amino acid is uncharged at physiological pH [129]. Furthermore, this transporter is considered to be specific for the L-enantiomer of BMAA. Although D-BMAA has been found in the mammalian brain, this compound is currently considered to be an enzymatic product formed within the CNS [67]. Given that essential amino acids require transportation across the BBB and that there are over 800 non-protein amino acids [130], is it conceivable that other non-incorporated, biologically active amino acids may also enter the CNS and have deleterious effects [131].

The identification of biologically active compounds in cyanobacteria continues to increase, through the identification of new classes of toxins and unique variants within a class. Included are many lipopeptides such as jamaicamides and antillatoxins that have neurological effects such as binding to sodium channels [84]. Although such channels are found throughout the peripheral nervous system (PNS) and CNS, further research may identify additional channels or transporters that allow them entry into the mammalian CNS.

4. Effects within the Peripheral and Central Nervous Systems

The compounds produced by cyanobacteria with neurotoxic potential are extremely diverse (Figure 1). However, such compounds generally affect essential systems within the cell. As previously mentioned, neurons represent a unique cell population in the CNS, and it is likely that certain neurons are particularly sensitive to cyanotoxin-induced damage and/or disruptions in cellular processes. Such convergent avenues for cyanotoxin-mediated cellular insults include:

4.1. Blocking of Essential Channels and Proteins

Compounds that block proteins and channels often result in rapid and severe effects in mammals. This mechanism is commonly observed for neurotoxins as well as frequently seen in acute intoxications. Cyanobacteria are capable of producing a range of protein blocking proteins such as estrogenic compounds and endocrine disruptors [81]. With respect to neurotoxicity, saxitoxins, antillatoxins and jamaicamides as well as non-cyanobacterial toxins such as tetrodotoxin, can block voltage-gated sodium channels resulting in a rapid inability to perform many neurological functions such as breathing [25,84,118,132]. Anatoxin-a binds to acetycholine receptors in competition with nicotine [133] and BMAA has the capacity to bind to NMDA and glutamate receptors [79,134,135].

4.2. Enzyme Inhibition

The screening of cyanobacteria has shown that small molecule eukaryotic enzyme inhibitors are common and such compounds include aeruginosins, anabaenopeptins, cyanopeptolins, microginins and microviridins (reviewed by [81]). A number of cyanobacterial toxins have been shown to be enzyme inhibitors, often of essential enzymes within mammalian cells. Microcystins (and nodularins) are able to inhibit protein phosphatases and phosphoprotein phosphatases [77,78], and this inhibition can be reversible or irreversible depending on the structure of the microcystin or nodularin variant [136]. Such

phosphatases are key cell cycle enzymes and, as these enzymes are located within the CNS, any inhibition may lead to neurotoxic effects [24]. Guanitoxin is an inhibitor of acetylcholine esterase, an essential neurological enzyme. Such enzymes are found within the CNS and remove acetylcholine from synapses [40]. Similarly, with protein phosphatases, inhibition of acetylcholine esterase could result in neurotoxicity [42].

4.3. Protein Damage

For proper cell function, the correct sequence and folding of proteins is required. If this process is affected, then significant deleterious effects can ensue. As a naturally-occurring non-protein amino acid, BMAA can enter cells and replace the non-essential amino acid L-serine in proteins [137]. Consequently, such misfolded proteins (even at low rates of amino acid misincorporation) may then act incorrectly within the cell and give rise to the potential for long-term damage. This scenario is especially relevant to the CNS where the reduction in toxic burden through dilution and cell-division is difficult [138].

5. Natural Intoxication Events and Methods for Cyanotoxin Evaluation

In order to understand whether a cyanotoxin may have neurological and/or neurotoxicological effects, appropriate bioassays need to be performed. In the case of known neurotoxins (e.g., saxitoxin and anatoxin-a), established in vitro and in vivo tests exist [139]. These include binding to acetylcholine receptors for anatoxin-a [25], acetylcholine esterase inhibition assays for guanitoxin [40] and saxiphilin binding assays for saxitoxins [140]. As the structures of many of the acutely toxic cyanobacterial toxins are known, ELISAs are increasingly being developed to screen for their presence in environmental and clinical matrices [141]. However, when required and during intoxication events, confirmatory methods such as mass spectrometry should also be used. Although there are many mass spectrometry methods to evaluate known neurotoxins such as anatoxin-a and saxitoxin (e.g., [142]), only one method has been developed for guanitoxin using hydrophilic interaction liquid chromatography (HILIC)–mass spectrometry [143,144]. The further development of such methods is essential. For example, they allow for the detection of specific neurotoxins when investigating human and animal health incidents and can confirm the results of high-throughput screening methods.

Beyond the detection of cyanotoxins by physicochemical methods such as mass spectrometry (Table 1), additional assays may also be necessary to understand cyanotoxin exposure and toxicity, as well as to determine what components of the mammalian cell are affected (e.g., enzymes or proteins). For example, while hepatocytes can be used to understand the hepatotoxicity of microcystins, assessments using neurological cell lines have further revealed the inhibition of enzymes that could explain microcystin neurotoxicity [120,121]. Historically, the mouse bioassay has been useful for assessing alkaloid cyanotoxins (e.g., [145]), deriving guidance through LD_{50} values and, in the case of guanitoxin, for observing hypersalivation and lachrymation [25]. However, due to ethical considerations, mouse model alternatives should be sought where possible. Invertebrate bioassays represent one useful alternative as they are highly sensitive to cyanotoxins and, due to increased numbers of test organisms, may also allow for robust statistical assessments. Examples of non-mammalian bioassays include the evaluation of anatoxin-a and saxitoxin using invertebrates such as *Artemia salina* [146] or *Daphnia* spp. [147].

Natural poisoning events involving acutely neurotoxic compounds have been demonstrated multiple times (Table 1). The poisoning of domesticated animals such as dogs from anatoxin-a [14] or guanitoxin poisoning of birds that consume *Anabaena* bloom material [12], all demonstrate the importance of bioassays. Although cyanotoxins may be relatively easily identified from such events, understanding the long-term issues of exposure is more difficult. Therefore, the selection of an appropriate assay and/or system is an essential part of understanding the relationship between cyanotoxin exposure and potential disease occurrence. Furthermore, for understanding long-term exposures, extensive research and statistical analyses are often required. For example, the relationship between

microcystin exposure and primary liver cancer was strengthened by analyzing microcystin concentrations in surface *versus* well water in China. Ueno et al. [46] showed that people who predominantly drank from well water were at lower risk of developing primary liver cancer than those who drank from surface waters potentially containing cyanobacteria. A similar relationship was observed in Eastern Europe with higher incidences of primary liver cancer in people who consumed water prepared from lakes with a history of known cyanobacterial blooms [148].

Table 1. Aspects of known cyanotoxins with neurological implications.

Toxin	Mechanism of Action	Poisoning Examples	Detection Methods	References
Saxitoxins	Inhibition of voltage gated sodium channels	h, d	LC-FD, ELISA, LC-MS	[149–154]
Microcystins	Inhibition of protein phosphatases	h, c, f, b, d	ELISA, EIA, LC-PDA, LC-MS	[76,155–160]
Anatoxin-a	Acetylcholine mimic	d, b	ELISA, LC-PDA, LC-MS, EIA	[43,158,161–165]
Guanitoxin	Acetylcholine esterase inhibitor	b, d	EIA, LC-MS	[12,16,42,144]
BMAA	Protein misincorporation, inhibition of protein phosphatase	h, do	LC-FD, LC-MS	[61,62,79,137,166]
Cylindrospermopsins	Protein synthesis inhibitor	h, c	ELISA, EIA, LC-PDA, LC-MS	[10,23,87,125,167–169]

h, human; b, birds; d, dogs; c, cattle; f, fish; do, dolphins; ELISA, enzyme-linked immunosorbent assay, EIA, enzyme inhibition assay; LC-FD, liquid chromatography–fluorescence detection; LC-PDA, liquid chromatography–photodiode array detection; LC-MS, liquid chromatography–mass spectrometry.

When used to complement epidemiological research, bioassays and model systems are fundamental to our understanding of natural intoxications or long-term exposures. The most recent example of long-term exposure to cyanobacterial toxins concerns BMAA, which was shown to be produced by freshwater and marine cyanobacteria and diatoms [54,56,57,59,170]. Through an assessment of locations with a high incidence of neurological disease, and combinations of specific neurological diseases such as ALS/PDC on the Island of Guam, BMAA and isomers have been identified as contaminating the traditional diet of Chamorro villagers [171]. Over 50 years of research on BMAA has shown that this amino acid is neurotoxic, present in the environment (including ancient pristine environments) and can be identified in brains of people who died of neurological illness [54,61,62,172,173]. Although these various lines of evidence are compelling in understanding the association of BMAA exposure and neurological disease, the biological assessment of BMAA toxicity has employed a wide variety of organisms including mice, rats, invertebrates and plants. Due to the wide range of toxic mechanisms ascribed to BMAA such as excitotoxicity, toxic metabolic products and receptor binding, many assays and systems have shown deleterious effects following exposure to this compound (reviewed by [174]). Of the mechanisms known, protein misincorporation is one possibility that could explain the development of neurological diseases such as ALS after BMAA exposure [175]. To this end, studies using non-human primates have shown that neuropathologies consistent with neurological diseases, including β-amyloid plaques, neurofibrillary tangles and microglial activation can be created after oral exposure [63,64].

Finally, another aspect of chronic cyanotoxin exposure is that often the effect of the purified toxin will be different or only partially account for the effect of the cyanobacterial extract containing the cyanotoxin of interest (e.g., [176]). Therefore, when assessing the potential for neurotoxicity some consideration of the chemical complexity of the extract may need to be considered.

6. Exposure Routes

Although the BBB is essential to protecting the CNS from pathogens and toxins [97], the actual route of cyanotoxin exposure may influence the toxicological outcome. Although

much is known about oral exposure through drinking water and contaminated food such as shellfish and dietary supplements, other routes of exposure include medicinal water (e.g., haemodialysis) and recreational exposure through practices such as water sports, showering and bathing, the washing of utensils and other personal objects in contaminated water and skin irritation [4]. During episodes of acute exposure, human health can be protected through practices including remediation measures such as drinking water treatment and the provision of alternative water sources to affected consumers. However, concerning chronic exposure, the various media and the durations of exposure when defining safe cyanotoxin concentrations is much more difficult. Although this has been carried out regarding tumor promotion in the case of microcystin-LR in drinking water (e.g., [177]), potential neurotoxic effects of compounds not classically considered to be neurotoxins may also need to be taken into consideration. Subsequently, such findings may affect future drinking water and bathing Guideline Values.

Increasingly, inhalation is being considered as a major exposure route. Application of cyanotoxins as sprays into the nose of experimental animals has indicated that this may increase the toxicity of certain toxins such as microcystins and anatoxin-a [178]. Furthermore, toxin analyses have shown that microcystins, BMAA and anatoxin-a can be recovered from filter material used in environmental and personal air sampling devices [179–182], and that the presence of BMAA, microcystins and potentially guanitoxin in desert crust may become airborne during dust storms [43,183]. Such studies highlight the potential importance of this exposure route. Due to the proximity of the nasal cavity to the human blood stream and brain, if cyanotoxins are inhaled then this may result in a more rapid uptake of cyanotoxins into the CNS, as they can travel along the olfactory nerve and bypass the BBB [184].

7. Synergism and Co-Exposure—Could This Be Significant?

In vitro investigations into the toxicity of cyanobacterial extracts containing known toxins suggest that compounds or components of these extracts can interact. As such, questions concerning the synergism and antagonism of toxins in cyanobacterial extracts need to be considered (e.g., [185]). Although acute neurological intoxications are unlikely to be affected by minor toxic components that are present within extracts, long-term exposure to such compounds may have an altered outcome due to synergistic and antagonistic interactions. As cyanobacterial compounds are rarely found in isolation, especially in complex media such as water or air, interactions are likely to occur. In addition to the presence of different cyanobacterial toxins such as LPS, hepatotoxins, cytotoxins and neurotoxins, other toxicants such as microorganisms, viruses, metals, pesticides, persistent organic pollutants, nanoparticles and plastics, as examples, may significantly affect the toxicological outcome of the cyanobacterial bloom material [70]. Ultimately, complex analyses of air and water are required in order to determine what combinations of toxicants are present. Multi-factorial toxicity assessments (e.g., [186]), using a variety of cell types and organisms will then allow the determination of what interactions may take place, in addition to providing information concerning what the permissible concentrations of cyanotoxins may be under various circumstances.

8. Future Needs and Requirements

Increasingly, evidence suggests that cyanotoxins not traditionally considered neurotoxic as well as cyanobacterial extracts that do not contain known cyanotoxins can exhibit PNS and CNS toxicity. To fully understand their neurotoxicity, assessments of cyanobacterial extracts may need to include such neurotoxic outcomes when both devising bioassays and when selecting appropriate cell lines and organisms. Furthermore, when future guidelines are derived, such neurotoxic properties may need to be taken into consideration in order to protect human health.

Historically, poisoning events, both animal and human have driven research into understanding the toxicity of cyanobacteria. Increasingly, newly discovered compounds

are found to have proven effects on neurological systems and the deleterious effects of other known compounds are being reviewed. Further analysis of human neurological disease cases occurring in proximity to harmful cyanobacterial and algal blooms may also be needed to fully consider their association. Ultimately, vigilance from medical practitioners and communication concerning the possible risks of cyanobacterial and algal blooms will help to protect human and animal health from the neurotoxicological effects of cyanobacteria.

9. Conclusions

Cyanobacteria are capable of producing a wide range of compounds with adverse effects in eukaryotic systems. Although toxins have traditionally been categorized based upon their primary mode of action, increasingly an ability of these compounds to have additional effects on neurological systems, of both the central and peripheral nervous systems is being recognized. Furthermore, the potential for cyanotoxins to have long-term chronic health effects is being acknowledged, in addition to the potential for synergism with anthropogenic and natural products, of cyanobacteria and contributed by other organisms in the environment.

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