Citation:
Stewart I (2010) Environmental risk factors for temporal lobe epilepsy – Is prenatal exposure to the marine algal neurotoxin domoic acid a potentially preventable cause? *Med Hypotheses* **74**(3):466-481.

Ian Stewart, Queensland Health Forensic and Scientific Services, 39 Kessels Road, Coopers Plains, QLD 4108, Australia; and School of Public Health, Griffith University, Logan campus, University Drive, Meadowbrook, QLD 4131, Australia
Tel.: +61 7 3274 9092; fax: +61 7 3274-9186
E-mail address: ian.stewart@griffith.edu.au

SUMMARY

Temporal lobe epilepsy with hippocampal sclerosis (TLE-HS) is one of the more common forms of chronic epilepsy. Its aetiology is unknown, though an early developmental insult is thought by some to be an important trigger. There is not a strong genetic predisposition; gene–environment interactions are more significant considerations. Environmental risk factors for TLE-HS are under-researched. Domoic acid (DA) is an environmental neurotoxin of algal origin that can contaminate marine food webs. DA can cross the placenta, is significantly more toxic to the developing brain compared to the adult brain, and has affected humans and marine wildlife through mass poisonings. DA coincidentally has a decades-long history of use as a chemical model of temporal lobe epilepsy, along with its close structural analogue kainic acid (also of algal origin). The principal hypothesis presented here is that dietary exposure to doses of DA that are sub-clinical in pregnant women may be sufficient to damage the foetal hippocampus and initiate epileptogenesis. The hypothesis could be tested both experimentally by *in vivo* proof-of-concept animal studies that expand on current knowledge of prenatal susceptibility to DA neurotoxicity, and by epidemiological investigations directed towards dietary exposure to marine food products. If only a small proportion of the attributable risk for TLE-HS is found to be due to gestational exposure to DA, the public health implications would still be of great significance, as this would represent a potentially preventable exposure.

Other potent neurotoxins are produced by marine microalgae and freshwater cyanobacteria. These structurally and mechanistically diverse toxins can also contaminate water supplies, seafood and shellfish. Several operate by modulating ion channels, so may also be of interest to epilepsy researchers.

DA is also the subject of preliminary scrutiny in strandings involving odontocete cetaceans. The implications of such work are discussed here.

Background

Epilepsy, temporal lobe epilepsy

Epilepsy is a neurological disease that is characterised by spasmodic and temporary interruptions to motor function, cognitive ability or both capacities concurrently. These events, known as seizures, are caused by bursts of aberrant electrical discharges in groups of cortical neurones. Seizures are diagnosed as epileptic when they have a tendency to recur spontaneously. Epileptic seizures can range from subtle, infrequent and relatively manageable events to sudden, dramatic and unpredictable paroxysms involving aberrant movement, loss of consciousness and urinary incontinence.

Epilepsy is a heterogeneous disease, affecting some 50 million people worldwide. The World Health Organization estimates that epilepsy accounts for an equivalent global burden of disease to breast cancer in women and lung cancer in men [1]. Epilepsy is the most common neurological condition of childhood, and the second most common – after stroke – in adults. Refractory temporal lobe epilepsy (TLE) is one of the common focal epilepsy syndromes in adulthood that results in great burden to patients and the community. There is limited knowledge of its aetiology. Prolonged febrile seizures resulting in hippocampal damage, and TLE secondary to cortical dysplasia or a tumour explain a proportion. The most common presentation of TLE, however, is hippocampal sclerosis (HS), or mesial temporal epilepsy [2]. The aetiology of HS is unknown, though much discussed in epilepsy research. A relationship between febrile convulsions and HS is being debated, with suggestions that early developmental effects are important, as HS in many cases appears to predate the onset of

febrile seizures [3]. Some discussion suggests that a prenatal insult may operate, particularly in the context of dual pathology, where extra-hippocampal brain pathology accompanies the typical pattern of HS, with the implication that the same developmental event is responsible for both lesions [4-8].

Kainoids and epilepsy research

Several unrelated chemicals are established research tools in animal models of focal epilepsy, e.g. pilocarpine, aluminium hydroxide [9]. Probably the most widely used chemical model of epilepsy is kainic acid (KA), a structural analogue of glutamate. Glutamate is the principal excitatory neurotransmitter in the mammalian central nervous system. Another close structural analogue of glutamate and kainic acid is domoic acid, which is the focus of this hypothesis. DA and KA act on a subset of glutamate receptors in the brain known as kainate receptors. Figure 1 shows the two-dimensional structures of DA and KA and demonstrates the close structural relationship to glutamate.

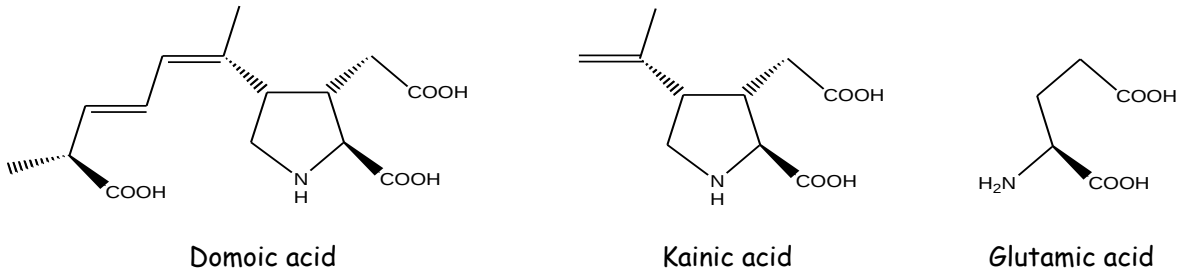

Fig. 1. Structures of kainic acid, domoic acid and glutamate

Kainic acid, and more recently domoic acid, are widely used chemical models of *status epilepticus* and temporal lobe epilepsy (many references, e.g. [10-13]). The clinical, histopathological, neuroimaging and biochemical changes seen after both experimental and natural exposures to DA are remarkably similar to the clinical findings of patients with hippocampal sclerosis, which is the most commonly presenting lesional substrate associated with temporal lobe epilepsy; TLE itself being the most commonly presenting chronic focal epilepsy disorder. Unilateral hippocampal microinjection of DA or KA in experimental TLE results in neuronal atrophy and gliosis in selective hippocampal sub-fields (CA1, CA3 and CA4, with sparing of cells in the CA2 stratum), and a seizure disorder that progresses over time, including a "silent" period during which the disease evolves – known as epileptogenesis [14,15]. These features are also prominent descriptors of the natural history of HS-related TLE, where very similar hippocampal pathology is recognised in neuroimaging studies and in post-surgical hippocampal histopathology, and a latent period is seen in which childhood seizures may resolve only to relapse years later into a chronic seizure disorder [15-17].

Epilepsy and natural exposures to domoic acid

Domoic acid and kainic acid were separately isolated from macroalgae in the 1950s [18]. In 1987, a common source outbreak of human poisoning caused by domoic acid occurred in Prince Edward Island, Canada; 107 cases were reported with symptoms of nausea, vomiting,

abdominal cramps, headache, diarrhoea and memory loss. The most severely ill subjects were hospitalised, 12 in intensive care. Fourteen patients showed persistent neurological dysfunction, four remained comatose and subsequently died. Specific neurological signs were seizures, myoclonus and severe anterograde memory loss. Positron emission tomography showed decreased glucose metabolism in mesial temporal structures. Brain histopathology of four subjects who died within 4 months of exposure showed neuronal atrophy and necrosis in the CA1 and CA3 hippocampal sub-fields [19]. Consumption of contaminated mussels was identified as the common exposure factor [19,20]. Subsequent investigations identified domoic acid as the toxic agent in the mussels [21], and a significant research endeavour then opened up as DA was found to be produced by marine microalgae known as diatoms (Class Bacillariophyceae). The term "Amnesic Shellfish Poisoning" was coined after this event, based on the anterograde memory deficits suffered by those most severely affected by the outbreak.

DA has since been implicated in several mass poisonings involving marine animals: confirmed DA-related mortalities have affected sea lions, seals and seabirds (pelicans, cormorants, shearwaters) [22-26]. Affected animals display neurological signs characteristic of DA poisoning: seizures, ataxia, repetitive head weaving, scratching and lowered response to external stimuli in sea lions; repetitive head movement, tremors, ataxia and vomiting in pelicans [25,26]. Autopsy findings in poisoned sea lions showed that the hippocampus was the target organ, with selective cellular vacuolation and necrosis in CA1, CA3 and CA4 strata, with cells in the CA2 region intact and relatively unaffected [25,27]. Domoic acid-producing diatom blooms are now regular events off the Californian coast, and trophic transfer of the toxin into California sea lions has allowed veterinary workers there to monitor and study chronic exposures and neurological outcomes. Bilateral asymmetric and unilateral temporal lobe pathologies are seen, as well as chronic intermittent epilepsies [28].

The proposed molecular mechanism of DA-induced epilepsy is that of excitotoxic injury and glutamate receptor sub-type activation in the brain [29]. That excitotoxic injury is an important consideration in the pathogenesis of epilepsy, including temporal lobe seizure disorders, was highlighted by Cendes et al. [30]. In a case report of an 84-year old man who survived the initial DA poisoning episode at Prince Edward Island, the authors describe an acute neurological illness including focal *status epilepticus*, which was controlled after 3 weeks with barbiturate pharmacotherapy. He became seizure free for a year and ceased medication; his electroencephalography investigations (EEG) were normal at 8 months after his initial illness. He then developed a focal seizure disorder; EEGs showed bilateral temporal lobe epileptic discharges and neuroimaging studies confirmed bilateral temporal and hippocampal abnormalities. He died of pneumonia 3¼ years after the initial exposure to DA. Autopsy findings showed total neuronal loss in the CA1 and CA3 sub-fields, sub-total loss in CA4, and moderate loss in the CA2 zone. Neuronal loss and gliosis was seen in other extra-temporal brain areas. This case study is an important illustration of the progressive nature of acquired temporal lobe epilepsy. A documented excitotoxic injury (in this case domoic acid by a natural exposure route) resulted in an acute neurological illness, including focal *status epilepticus*, which then resolved (the silent period). Temporal lobe epilepsy subsequently manifested. The hippocampus was the most severely affected brain structure, as implied by the clinical presentation and post-mortem findings, and (though this aspect was not discussed by the authors) the extra-temporal brain lesions are not inconsistent with the concept of dual pathology resulting from the same earlier brain insult. The authors describe their case study as "strongly support[ing] the role of excitotoxic injury in epileptogenesis." [30].

Experimental and observational evidence for increased foetal and neonatal sensitivity to DA

DA is reportedly 40 times more potent on a weight basis to neonatal rats than adult rats [31]. Neurotoxicity of DA was shown to decrease progressively with increasing age of exposure in

neonatal rats [32]. Chronic cognitive deficits were reported in rats exposed prenatally to a single dose of DA [33]. Altered cognition was also seen in adolescent rats exposed to low doses of DA as neonates, though prenatal exposures reportedly result in more widespread and long-lasting adverse effects [34]. Long-term alterations in learning and memory capacity were demonstrated in rats exposed to serial low-dose injections of DA over a 1-week period as neonates; some enhanced abilities were seen, with increased hippocampal mossy fibre sprouting posited as an explanation [35]. Low-dose DA administered to neonatal rats has initiated seizure-like responses in adulthood in response to external stimuli (i.e. the "seizures" were not seen to occur spontaneously but appeared to be evoked by novel environmental stimuli) [36]. Male C57LB/6 mice exposed prenatally to DA at 1 mg/kg i.p. reportedly demonstrated chronic behavioural abnormalities, and immunohistochemical markers revealed persistent cortical myelin decrements and cortical and hippocampal dendritic overgrowth. No hippocampal cell loss was seen; it is not clear from the report why only male offspring were investigated [37]. DA can be transferred experimentally to rat pups through milk, thereby establishing potential natural exposure routes in early development [38]. Explanations as to why the developing brain appears to be more susceptible to the neurotoxic effects of DA include the incomplete development of the blood–brain barrier [39], reduced renal clearance [31,38] foetal-specific exposures due to dermal and gastro-intestinal absorption from amniotic fluid [40] and age-dependent proportional differences in glutamate receptor sub-type and/or differential maturation of excitatory and inhibitory neurones [32]. DA, like other weak acids, can cross the placenta and in experimental studies has been identified in foetal brains and amniotic fluid [41]. Amniotic fluid is posited as a sink for DA, with delayed keratinisation and late development of the stratum corneum being mechanisms that facilitate the partitioning of water-soluble toxins such as DA across the foetal skin [40]. Amniotic fluid is swallowed during late gestation; observational studies in DA-affected sea lions showed similar concentrations of DA in amniotic fluid and foetal gastric fluid, suggesting a further route of sustained exposure *in utero* [40].

Domoic acid as an environmental neurotoxin

As discussed above, domoic acid and its kainoid receptor agonist analogue kainic acid have a history of use in basic neuroscience and epilepsy research as glutamate analogues and *in vivo* models of temporal lobe epilepsy. However, a parallel public health research effort has developed since the 1987 human poisoning incident in Canada. Harmful algal bloom (HAB) researchers have learned much about the ecology and distribution of domoic acid-producing microalgal blooms, even though this aspect of DA research is still very much in its infancy. *Pseudo-nitzschia*, the principal DA-producing diatom genus, has a cosmopolitan distribution [42,43]. Some twelve *Pseudo-nitzschia* species are capable of producing DA [44], as does the benthic diatom *Nitzschia navis-varingica* that was recently discovered in a shrimp aquaculture pond in Vietnam, and since found to be widely distributed in brackish waters [45]. A near real-time sweep for a range of structurally unrelated HAB toxins in the North Sea found DA to be ubiquitous at all sampling locations, though with a wide concentration range [46].

Public health responses to DA have focused on the risks associated with direct consumption of DA-contaminated seafood. Regulatory limits of 20 μg DA per gram of shellfish flesh have been adopted by food safety agencies internationally; shellfish harvesting closures result when this level is exceeded [47,48]. This action level for DA of 20 μg/g was derived from a retrospective assessment of DA in mussel tissue that was associated with illness in the Prince Edward Island outbreak (200 μg/g mussel flesh), and incorporates a single 10-fold safety factor for inter-individual variability (intra-species variability in humans) [47,49].

While DA levels in shellfish have been and continue to be the main focus of attention for HAB researchers and monitoring agencies in North America and Europe, the potential exists for more diffuse entry of DA into the food chain. Trophic transfer of DA is known to occur

through consumption of contaminated planktivorous fish (sardines and anchovies), as evidenced by investigations into marine animal poisonings [22-26]. During an investigation of a diatom bloom and related sea lion mass mortalities off California in 1998, mussels (*Mytilus edulis*) were found to be negative or to have only trace levels of DA, whereas anchovies and sardines caught during the bloom period had levels ranging from 30 to 110 μg/g [25]. DA has been found in various molluscan bivalves: mussels, razor clams, cockles, scallops [47,50], crustaceans – several crab species [47,51,52] and tunicates [53]. DA has the capacity to move extensively through marine food webs, being found in blue whale faeces, demersal and pelagic fish, e.g. mackerel, albacore tuna, turbot, sole, halibut [54,55]. Shellfish and crab fishery closures due to excess DA levels have occurred in the USA [56], Canada [48], Scotland and Ireland [50], Spain [57] and Mexico [58]. DA-producing diatom blooms are now a regular occurrence off the coast of California [24]. High levels of DA have been detected in scallops from New Zealand: 210 μg/g in whole scallops and 600 μg/g in viscera [48].

Algal biotoxin monitoring in Australia varies across different state agencies, with regular sampling for *Pseudo-nitzschia* spp. in South Australian and Tasmanian oyster and mussel fisheries. Queensland has a limited monitoring program covering four oyster-harvesting areas in Moreton Bay. A mussel fishery was briefly closed in Port Lincoln, South Australia in 2004 due to excess *Pseudo-nitzschia* cell counts, and DA was detected in mussel flesh at 0.5 μg/g (Clinton Wilkinson, SA Shellfish Quality Assurance Program, personal communication, 3rd September, 2009). A research screening survey for several harmful algal toxins identified DA at low levels in Moreton Bay, Queensland [59].

Hypothesis: that a proportion of attributable risk for temporal lobe epilepsy of unknown aetiology may be explained by prenatal exposure to domoic acid

Domoic acid is a more potent excitotoxin than kainic acid when used as an experimental model of TLE. Natural exposures to DA have caused chronic TLE in an elderly man, and seizure disorders and hippocampal pathology in sea lions. DA can cross the placenta. Food safety monitoring for DA is limited to certain bivalve shellfish and some crab fisheries, and is not done comprehensively within and between nations. The distribution of DA in finfish that are caught and sold for human consumption is essentially unknown. There is a growing body of evidence that suggests that the developing brain is more sensitive to the neurotoxic effects of DA. Single-dose exposures to DA, seen both experimentally and by natural exposure, can cause chronic epilepsy.

This hypothesis proposes that a proportion of the attributable risk for human temporal lobe epilepsy of unknown aetiology may be explained by placental transfer of low doses of domoic acid present in the diet of women consuming seafood or shellfish at critical periods during their pregnancy. Such doses would generally be below the threshold that induces vomiting in the mothers.

An unknown early life insult is implicated in the aetiology of a significant proportion of TLE cases that are not explained by identified environmental factors such as trauma or tumour [1]. The experimental research findings of increased susceptibility to DA in the prenatal and neonatal periods, as discussed above and recently reviewed by Pulido [60], document various experimental endpoints, including hippocampal pathology, EEG abnormalities and memory-related cognitive dysfunction. However, the most interesting finding relating to the potential for DA to initiate TLE is arguably that of Dakshinamurti et al. [61]. These workers exposed mice prenatally to a single low dose of DA, and demonstrated progressive histopathological and electrophysiological changes in the hippocampus of growing pups. No hippocampal damage was seen in pups sacrificed on day 1, severe damage to the CA3 sub-field and dentate gyrus was seen at day 14, and CA4 involvement was seen at day 30. Diffuse spike and wave

activities (i.e. epileptiform discharges) were seen at day 30. No overt seizures were observed in pups, and the experiment was completed at day 30. The authors describe these findings and other experimental work on various brain biochemistry parameters in their report as indicative of excitotoxic damage to the hippocampus caused by mid-gestational exposure to DA. While this is an entirely reasonable conclusion, these findings can also be interpreted as diagnostic predictors of either an existing but unrecognised spontaneous temporal lobe seizure disorder in their mice, or a developing but latent TLE that did not manifest due to early termination of the experiment.

Spontaneous, intermittent focal epileptic seizures in mice are difficult for the inexperienced or occasional observer to identify. In humans, epilepsy is most often a clinical diagnosis, made from the presenting history and description of seizures by a relative or witness such as a schoolteacher, and supported by EEG and neuroimaging studies, neurological examination and a favourable response to anti-epileptic pharmacotherapy [62]. Many neurologists may never actually witness a seizure in most of their patients with epilepsy, particularly in those whose disease is well controlled. However, for patients in whom the diagnosis of epilepsy is uncertain, and for those with unequivocal epilepsy who are being assessed for epilepsy surgery (where more detailed information about seizure semiology is required by clinicians), specialised clinical laboratory investigations are conducted. The principal technique here is that of EEG video telemetry, where the patient is monitored continuously by a video-camera while EEG data is concurrently recorded. The study can be conducted over several days, so that intermittent seizures, whether brief (measured in seconds) or prolonged (minutes) are captured on video and can be examined and re-examined in detail, along with concomitant EEG data [63]. Video recording and EEG acquisition are also used to confirm the diagnosis of seizures in laboratory animal models of epilepsy, e.g. [64,65]. Epilepsy models in small rodents are best conducted in laboratories with such specialised equipment and expertise, otherwise experimentally induced seizures may be overlooked.

Dakshinamurti et al. [61] did not appear to systematically monitor their experimental animals for the occurrence of intermittent seizures; they did not report use of video monitoring or intensive observation during awake periods for nocturnal rodents (i.e. night-time if normal day/night lighting cycles were used, or the use of reverse day–night lighting phases). These comments should not be interpreted as criticism of Dakshinamurti et al. for failing to systematically monitor for spontaneous seizure generation. Their study was a time-sequence design, where pups exposed prenatally to DA underwent EEG recordings under anaesthesia, after which they were sacrificed on postnatal days 10, 20 and 30, and brain tissues were removed for histopathological examination. These experimental endpoints are different to those that would be required in a study of spontaneous seizures following mid-gestational exposure to domoic acid; besides which, a study to identify chronic epileptic seizures following single-dose prenatal DA exposure would need to continue for longer than 30 days in order to allow further progression of epileptogenesis. Rats have been shown to develop spontaneous seizures 3–4 months after single intra-hippocampal injections of kainic acid [66]; the proposal for this proof-of-concept mouse model is for a 3-month post-natal study period. The work of Dakshinamurti et al. [61] has demonstrated interesting findings of hippocampal pathology and temporal lobe EEG abnormalities that invite further studies designed to explore the possibility of spontaneous seizure generation following low-dose mid-gestational exposure to DA.

Further study design enhancements to explore the reported hippocampal and electrophysiological abnormalities caused by low-dose mid-gestational exposure to domoic acid might include investigator-blinded examination of the brain structure and function of dosed and control dams (Dakshinamurti et al. did not describe hippocampal histopathology or EEG studies of dams in their report), and collection of urine from dams after intravenous administration for quantification of DA so that experimenters can be confident that DA doses were effectively delivered. Blinding of investigators at all stages of the experiment (dosing,

observation for spontaneous seizures, EEG and histology) is a feasible technique to enhance the robustness of the design if the same mouse strain is used for test and control groups. Confirmation that doses causing pathological changes in offspring did not affect dosed dams would provide proof-of-concept for the hypothesis that dietary exposures to domoic acid that are sub-clinical for adults may be neurotoxic to the developing foetus. Further proof-of-concept might involve repeat studies employing oral exposures in a vomiting-capable model such as cat, dog or ferret, as small rodents do not vomit. The ferret is reportedly very similar to humans in its emetogenic response [67].

Epidemiology of domoic acid exposure

Epidemiological investigations into dietary exposures to domoic acid are very limited. This is partly explained by the fact that the first and so far only documented outbreak of DA intoxication in humans occurred just 22 years ago [20]; before this event DA was not recognised as an environmental toxin. High DA levels in razor clams (*Siliqua patula*) from Washington and Oregon (USA) in 1991 triggered recreational and commercial harvest closures [68]. Gathering these shellfish has been a customary practice of Native American populations in the area; such activities may place these people (and other indigenous groups elsewhere in the world) at greater risk of DA intoxication because wild harvesting is often outside the aegis of regulatory interventions such as monitoring, depuration and harvesting restrictions. Epidemiological investigations have been initiated to study the risks of exposure to DA in these groups [55,69].

The domoic acid poisoning incident at Prince Edward Island in 1987 led to public health investigations and subsequent toxicological studies to trace the source of the outbreak [19-21]. The subsequent development of TLE in an elderly victim of the outbreak was presented in a case report, as discussed above [30]. Otherwise, no prospective or retrospective studies have been published that examine environmental exposure to DA as a risk factor for epilepsy in humans.

By contrast, veterinary and HAB researchers are actively investigating the epidemiology of domoic acid exposure and its relationship to the development of epilepsy in California sea lions (*Zalophus californianus*). Reports of an initial mass poisoning in 1998 describe acute neurological signs and findings of domoic acid intoxication in adult animals: ataxia, head weaving, convulsions and *status epilepticus*, lethargy. Focal convulsive seizures were described. Seizure control was achieved with parenteral benzodiazepine and oral barbiturate therapy. The hippocampus displayed the most obvious neuropathology, with necrotic changes affecting (in decreasing order of severity) CA3, CA4, CA1 and CA2 sub-fields. Post-necrotic hippocampal atrophy and gliosis was observed in sub-acute cases. Pathology of extra-hippocampal limbic structures was seen in some animals, in the pyriform lobe, ventral rhinencephalon and thalamus [23,25].

Domoic acid-producing *Pseudo-nitzschia* blooms occurring in the following years allowed veterinary workers to continue their observational studies of neurointoxication in sea lions, documenting structural and functional neuroimaging findings of temporal lobe pathology, histopathological, electroencephalographic and behavioural abnormalities that will be familiar to clinicians working in the field of human epilepsy. Asymmetrical bilateral hippocampal pathology, unilateral hippocampal atrophy – detected by magnetic resonance imaging (MRI) and at necropsy – decreased glucose uptake in mesial temporal structures by positron emission tomography, and focal and generalised epileptiform changes on EEG were described. The epidemiology of epilepsy in California sea lions may be changing over time, with increasing frequency of domoic acid-producing diatom blooms over the last decade. Sub-acute DA poisoning cases have been shown to develop chronic epilepsy, and the demographic profile of neurologically abnormal animals appears to have changed over the

period since observation of these domoic acid intoxications began in 1998. Adult female sea lions were most often seen then, suffering acute signs of domoic acid intoxication, whereas there are now higher proportions of juvenile animals of both sexes suffering chronic complex partial seizures. This suggests evolving and progressive seizure disorders initiated by pre-natal exposure to sub-lethal concentrations of domoic acid, possibly augmented by further neonatal exposures when suckling [27,28]. The hypothesis that trans-placental exposure to domoic acid is an important consideration for observed neurological impairment in California sea lions is explored further by Ramsdell and Zabka [40] in their review of experimental and observational research.

Epidemiology of epilepsy and temporal lobe epilepsy

The modern understanding of epilepsy is in no small measure shaped by the findings of a diverse body of epidemiological research. Early studies in the 1960s and 1970s led to the adoption of some important diagnostic criteria and the recognition of epilepsy as a disease characterised by recurrent seizures. Symptomatic and idiopathic epilepsies and the various epilepsy syndromes were subsequently described. Geographic influences on the aetiology of some symptomatic epilepsies were revealed, particularly regarding central nervous system (CNS) infectious agents such as tuberculous meningitis, AIDS-related toxoplasmosis and neurocysticercosis [62]. Studies into secular trends, the natural history of various epilepsies, descriptive epidemiology and genetic epidemiology have been conducted, as well as a good number of prevalence and incidence studies [70-72]. Epidemiological investigations are also applied to the assessment of pharmacotherapies and surgical treatments, and the study of various comorbid conditions. This broad scope of epidemiological endeavour reflects the understanding that epilepsy is a heterogeneous disease, with a correspondingly broad span of aetiological influences, diagnostic criteria, medical, surgical and socio-cultural interventions and prognostic expectations [72]. Yet while the epidemiological investigation of the epilepsies has been multifaceted and extensive, there are some epidemiological questions that have not yet been applied to the study of this disease. These questions, in particular those related to maternal dietary exposures, will be discussed below under "Proposed testing of the hypothesis."

This hypothesis is specifically concerned with temporal lobe epilepsies, particularly TLE with hippocampal sclerosis. The epidemiology of TLE is incompletely understood. TLE is the most common type of focal epilepsy treated at specialist epilepsy surgery centres across the world, although such findings suffer from biases towards patients who are surgical candidates, have more severe seizure disorders and are able to access comprehensive diagnostic facilities [73]. Because of the difficulty in applying strict diagnostic criteria to general practice and population-based epidemiological studies, especially those pertaining to localisation of focal epilepsies, the actual frequency of TLE in the population is unknown [73]. Nevertheless, there is a general consensus amongst epileptologists that TLE is the most commonly encountered of the chronic focal epilepsies. High proportions of TLE cases are cryptogenic, in which a pathological substrate is suspected but unidentified, and TLE with hippocampal sclerosis, for which the aetiology is unknown, also represents a significant proportion of the overall prevalence of TLE [2].

Proposed testing of the hypothesis

The proposal here is for a combined approach incorporating experimental *in vivo* neurotoxicology studies, and observational epidemiological investigations to identify associations between maternal dietary exposures and temporal lobe epilepsy. Successful animal model/s of spontaneous seizure generation following mid-gestational exposure to

domoic acid would represent proof-of-concept; such an eventuality would presumably lend support to research enquiries into the epidemiology of cryptogenic TLE and TLE-HS.

Proof-of-concept mouse model

As discussed above, the study of Dakshinamurti et al. [61] was not designed to elicit epileptic seizures in their mouse model. However, their discovery of progressive excitotoxic damage to hippocampal structures is suggestive of some pathological substrates that may underpin the subsequent development of intermittent temporal lobe seizures. A pilot study to determine appropriate sample sizes could be followed by an experiment using exposure conditions (doses, exposure route, timing) and detailed histopathological examination of hippocampal structures similar to those described by Dakshinamurti et al. [61]. Modifications to the study design would involve extending the study until exposed pups and controls are 3 months of age; use of video monitoring to ensure that intermittent seizures, should they be initiated in the exposed group, are observable by study workers; serial acquisition of EEG data; confirmation of systemic administration by measuring domoic acid in the urine of dosed dams; application of study methods to dosed dams as further controls (for proof-of-concept that doses of domoic acid that are sub-toxic to pregnant females can initiate excitotoxic damage to the offspring); and adoption of an investigator-blind design.

Epidemiological hypothesis testing

Epidemiological investigation of the hypothesis could be approached in a number of ways. A preliminary data-gathering approach might involve an ecologic study; this study design has groups of individuals as the unit of observation. In this case the unit of observation would be at the level of countries. Such a study could compare national seafood consumption statistics and epilepsy disease registries to investigate whether countries with higher dietary seafood intake have a correspondingly increased prevalence of TLE-HS and cryptogenic TLE.

Descriptive epidemiological information could be gathered from mothers who did not consume seafood during pregnancy, to compare prevalence of TLE-HS in their offspring with that of the broader population. These women could be recruited through State and National vegetarian and vegan societies and similar organisations.

More robust epidemiological approaches might include case–control studies to elicit information on seafood intake during pregnancy by mothers of TLE-HS cases. While recall of dietary habits during pregnancies several years or even decades prior to interview would often be vague at best, ordinal scale estimates of seafood and shellfish consumption (none, low, medium, high) may be obtainable. And finally, when large-scale prospective cohort studies are eventually conducted for cryptogenic TLE and TLE-HS, as has been called for by other workers [73], the current body of literature on domoic acid – a known epileptogenic excitotoxin present in marine food webs, to which the developing brain appears to be at increased risk – should warrant inclusion of gestational seafood exposure estimates.

Domoic acid in seafood and shellfish: studies to refine exposure assessments

Toxic *Pseudo-nitzschia* blooms, like other HAB events, are ephemeral, localised and intermittent, so the occurrence and concentrations of domoic acid in seafood and shellfish, while very significantly under-researched, are likely to be spatially and temporally heterogeneous. Routine monitoring of edible marine products is limited to (mostly) bivalve molluscs, and monitoring intensity is variable within and between nations (for example, in Australia, shellfish exported to the European Union appear to be more comprehensively

screened for domoic acid than shellfish from domestic fisheries). Opportunistic sampling of bivalve molluscs for research investigations not directly related to food safety issues has revealed contamination by domoic acid: 11 of 12 blue mussel (*Mytilus edulis*) samples purchased from retail outlets in Taiwan were found to contain DA [74]. Random sampling of a range of bivalve molluscs from coastal waters in the Philippines, Thailand, Vietnam and Japan revealed widespread presence of DA at a broad concentration range, with specimens of the genus *Spondylus* accumulating quantities well above current alert levels [75]. *Spondylus squamosus* (thorny oyster) also appears to be a very efficient saxitoxin accumulator [76], which suggests this seafood product should be subject to particular scrutiny from a food safety perspective in this regard (see below for discussion of other HAB toxins). Curiosity-driven ecotoxicology studies on Stradbroke Island, Queensland, Australia, detected DA in each of three shellfish species sampled: wild mussels (*Modiolus proclivis*), oysters (*Saccostrea glomerata*) and pipis (*Donax deltoides*) [59]. Wild pipis, and to a lesser extent mussels and oysters, are regularly harvested by the island's traditional owners (Quandamooka community), other Aboriginal and Torres Strait Islander residents and recreational fishers. While useful inferences obviously cannot be drawn from such a small sample base, the observation that DA can so easily be found in shellfish available for human consumption indicates that systematic food safety studies are warranted.

Domoic acid has been identified in a wide range of marine food products, including finfish (see above), but these research findings have not yet been translated into routine public health surveillance of a wider range of seafoods. A systematic survey of a broad range of edible marine products has not been conducted to date; an international collaborative survey to detect and quantify domoic acid may be a suitable approach. Targeted sampling could investigate high risk foods: planktivorous fish (particularly Clupeids), bivalves and crabs, as well as higher-risk practices (recreational crab fisheries, wild-harvested bivalves). Seafood and shellfish export markets are widespread and growing; a broad international survey, repeated over several time intervals, will provide a much clearer understanding of the distribution of domoic acid in marine foods. Regional and/or temporal variability in the distribution of DA in seafood and shellfish can be anticipated: a recent study of retailed shellfish in South Korea identified DA in only 0.5% of nearly 900 samples [77]. DA levels in *Spondylus versicolor*, a popular edible shellfish in Vietnam, exhibited significant temporal variability when examined over a 10-month period; although low background levels of 1–2 μg/g DA were detected throughout the year, spikes of up to 150 μg/g were found [78]. DA was detected at concentrations below 20 μg/g in "almost every bivalve species all around the Portuguese coast for short periods scattered in time and coincident with the occurrence of *Pseudo-nitzschia* spp." in 1996 [48].

Caveats and challenges. 1: animal models of gestational exposure to domoic acid and development of TLE

Animal models of spontaneous epileptic seizures have in the main been conducted using adult animals, with a smaller proportion investigating juveniles and neonates. This seems understandable insofar as *in vivo* models are used to investigate one or a few of a wide spectrum of exposure and intervention parameters (e.g. genetic influences, molecular mechanisms, pharmacotherapies, pathological substrates, seizure-related neurogenesis). Using juvenile or adult animals offers convenient access to whole-animal biological systems, and success or failure of the experiment can usually be determined fairly soon after the seizuregenic exposure is administered. The proposition arising from this hypothesis is for a rodent model to test a presumed natural exposure to a known excitotoxin during mid-gestation, albeit initially by a parenteral exposure route. The proposed model would not investigate an isolated biological mechanism operating within a whole-animal system. Rather it is a proof-of-concept approach that utilises the complexity of a whole-animal system, from trans-placental exposure and toxicokinetics, to the pathological effects of a seizuregenic toxin

on the mid-gestational brain, progressive development of excitotoxic hippocampal injury (and possibly extra-hippocampal pathology) through to the eventual outcome of spontaneous intermittent temporal lobe seizures. The proposed model is therefore less of a reductionist approach in this instance, more an investigation of interconnecting, emergent processes. No criticism of the large body of mechanistic neuroscience research into epilepsy is implied here; indeed many aspects of the current knowledge base (e.g. excitotoxic injury and epileptogenesis, electroencephalography) would need to be drawn upon in order to test the hypothesis.

So a significant stumbling-block for an animal model of gestational initiation of adult TLE-HS is the time interval required before experimental success or failure can be determined. Waiting out the remainder of the prenatal period after dosing, then waiting through the development of a silent period before seizures manifest will place significant demands in terms of researcher time and animal agistment costs. However, if an unknown developmental insult is considered an important aetiologic factor in a significant proportion of TLE-HS cases, as some epileptologists and neurologists consider to be the case (see above), then an argument can be made for *in vivo* modelling of this complex phenomenon, including seizuregenic exposures other than the focus of this particular hypothesis. Design and operation of such studies would draw on the expertise of researchers familiar with gestational toxicology methods as well as epilepsy researchers familiar with detecting spontaneous seizures in laboratory animals (these events can be subtle, particularly in mice, and may be overlooked by workers lacking the requisite experience and equipment).

Kainic acid and domoic acid are widely used as chemical models of TLE for *in vivo* and *ex vivo* studies. Their utility for modelling TLE is described in terms such as: "This rat model reproduces many of the pathologic, behavioural, and electrophysiologic features of human mesial temporal lobe epilepsy…" [79]; "we describe … histological, electrophysiological and behavioural evidence that discrete excitotoxic lesions of the hippocampus in mice represent a valid model of human MTLE." [80]. However, such opinions are not universally acknowledged. Jay and Becker [81] stated that:

> "…animal models [of] stimulation of the perforant pathway, selective kainate toxicity of neurons, or ischemia … have failed to replicate the changes of classic hippocampal sclerosis."

Yet (at least in the case of exposure to the kainate neurotoxins), the opinion of Jay and Becker may reflect the fact that the large number of experimental studies reported to date, with the exception of that by Dakshinamurti et al. [61], were conducted on adult or post-natal animals. Sloviter [82,83] critiques the exposure conditions in many animal models of TLE, particularly the initiation of epileptogenesis following prolonged *status epilepticus*. Such conditions do not represent the clinical presentation and history of human MTLE-HS; Sloviter calls for *in vivo* experimental designs that better model the development of epilepsy in humans [82,83]. In order to study the effects of the putative early life environmental insult thought to explain an unknown proportion of TLE-HS cases, *in vivo* models that incorporate foetal brain development should be designed. Exposure to excitotoxic injury during a discrete gestational window when massive apoptotic cellular turnover, neuronal migration and synaptic organisation and reorganisation are occurring may present a different histopathogical and functional profile to that of juvenile and adult exposure to kainate excitotoxins.

The pharmacokinetics of kainic acid and domoic acid administered trans-placentally are much less understood than is the case with parenteral or even intra-hippocampal dosing in juvenile and adult animals. However, the pioneering work of Dakshinamurti et al. [61] together with the observational studies arising from the USA on domoic acid intoxication in sea lions [40] shows that DA can cross the placenta and injure the developing brain.

Caveats and challenges. 2: epidemiology of exposure to domoic acid

Reliably determining exposure
Using dietary seafood intake as a proxy for exposure to domoic acid is likely to be unreliable in the absence of comprehensive surveys for DA concentrations in edible marine products. While *Pseudo-nitzschia* spp. have a cosmopolitan distribution, and limited sampling programs have found domoic acid to be widely distributed in marine waters, DA concentrations in seafood and shellfish are likely to be highly variable both temporally and spatially. Studies into DA in commercial and wild-harvested marine-origin food products should precede planning for costly epidemiological investigations (i.e. anything beyond an ecologic study as discussed). Systematic sampling and testing for DA in seafood is now technically feasible, using a commercially-available enzyme-linked immunosorbent assay (ELISA) as a screen, with confirmatory analysis using (say) a high performance liquid chromatography + tandem mass spectrometric method.

Other seafood-related variables may complicate epidemiological investigations into developmental risk factors for TLE-HS, for example methylmercury exposure. Methylmercury can disrupt ion channel function and neuronal migration [84,85], so epidemiological designs to investigate prenatal exposure to kainate excitotoxins through maternal seafood exposure would need to accommodate the potential for interaction with such extraneous variables.

Recruitment
A knowledge and treatment gap for epilepsy exists in many developing countries [86,87]. Precise diagnostic criteria and (often) sophisticated imaging techniques are needed to identify MTLE-HS and cryptogenic TLE cases. Varying capacities to diagnose, classify, document and disseminate information to researchers about these specific, albeit common forms of chronic epilepsy will impact negatively on international epidemiological investigations, including ecologic studies. Some developed countries have dedicated epilepsy disease registries that support recruitment into epidemiological studies [88,89]. Recruitment of TLE cases in countries without such facilities would require traditional door-to-door household surveys.

Domoic acid in marine food webs: some supplementary questions

The understanding that DA is available in marine food webs raises some disparate questions that may contribute to the broader public health enquiry into this under-researched environmental neurotoxin. Some of these are raised below, in no particular order:

Could dietary exposure to DA explain some mass whale strandings?

Researchers in the USA and Mexico have detected DA in tissues and faeces of baleen whales [54,90,91] and toothed whales [91-93]. Temporal correlations between toxic *Pseudo-nitzschia* blooms off the Californian coast and mortalities involving several dolphin species have also implicated domoic acid poisoning as a likely explanation [91]. Other workers suspect domoic acid may be responsible for some whale and dolphin mortalities they have investigated, and a growing volume of internet traffic is flagging these suspicions, e.g. [94-96]. The 2004 mass die-off of bottlenose dolphins in the Florida Panhandle was subsequently attributed to brevetoxin poisoning [97,98].

The presence of DA in sperm whales and dolphins is particularly interesting as the toothed whales (suborder Odontoceti) encompass those species that suffer periodic mass stranding.

The causes of these dramatic phenomena remain unknown, despite much speculation and intensive research efforts. Some hypotheses seem to be uncertain at best, e.g. decompression sickness due to rapid escape from naval sonar and petroleum exploration activities [99]; mass strandings long predate such activities. The widely-investigated topic of persistent organic pollutants (POPs) and organometals in cetaceans has contributed significantly towards the understanding of the uptake and distribution of lipophilic xenobiotics in marine systems. But chronic and incremental exposure to POPs would appear to be a weak explanation for sudden and catastrophic mass strandings. The continuing research effort into POPs and heavy metals in cetaceans – insofar as it is directed towards explaining mass strandings, at least, e.g. [100-104] – might be construed as an example of what the food writer Michael Pollan calls "parking-lot science," the allegory of a man who loses his keys in a car park at night. He looks for them under the sodium lamps, aware that he probably did not lose his keys there but looking anyway because that's the only part of the lot where he can see clearly [105]. Blubber and other peripheral tissue samples are easily obtainable from stranded whales, and fairly small samples can simply be frozen and transported for analysis of a wide range of fat-soluble anthropogenic compounds. Confirmation of domoic acid-related mortality would require detection and quantification of a water-soluble biotoxin in tissues such as stomach contents, urine, faeces, blood, milk and amnion; removal and fixation of intact brains soon after death in order to facilitate detailed histopathological examination; and perhaps expert identification of *Pseudo-nitzschia* in the stomach contents of prey fish and cephalopods taken from stomachs of stranded animals. Such activities would require a significant degree of forward planning, and could not be approached with anything like the ease and convenience of field sampling for lipophilic toxin analysis.

Two decades of observational and experimental research have shown that the kainate excitotoxins can cause acute cognitive dysfunction. Yet the proposition that mass strandings may in some cases be caused by the pod feeding on DA-contaminated schooling prey has received very little attention. The biological plausibility for sudden and calamitous behavioural change in large cohorts would appear to favour acute neurointoxication over chronic accumulation of lipophilic toxins. Of course, isolated findings of domoic acid in the tissues of stranded whales do not imply causation, but these discoveries may invite more cross-disciplinary collaborations to actively investigate a domoic acid and mass stranding hypothesis. Some research activities to augment these findings might include developing tools to assess cognitive function in stranded animals; histopathological and volumetric studies of brain structures (especially the hippocampus); acquisition of EEG data from living stranded whales and dolphins. These would not be trivial exercises; considerable organisational challenges and funding support would be required. Yet there are recent technical and knowledge-base gains that could facilitate some of these proposals: advances in miniaturisation have allowed the development of laptop-based portable EEG systems (though again, detailed planning and organisation would be required in order to rapidly transport such a device and an operator to stranding sites, as well as meeting technical challenges in acquiring EEG data from skin that needs to be kept moist). Procuring whole brains for histopathological examination in suspected DA-related strandings may require significant forward planning, as the cetacean hippocampus is a very small structure and not easily identified in the absence of other cortical landmarks [95] (see below for discussion of the cetacean hippocampus).

Whale and dolphin strandings are complex phenomena that may occur for different reasons. But dietary exposure to domoic acid is arguably worthy of a coordinated, multi-disciplinary research investigation. A recent report in the mainstream press suggested that a large pod of dolphins were behaving as if disoriented, with expert opinion suggesting injury to the animals' sonar system as the likely explanation [106]. Acute cognitive dysfunction might also be considered as an alternate hypothesis.

Could kainic acid also be present in marine food webs?

Kainic acid and domoic acid were isolated from marine macroalgae in the 1950s; these seaweeds have a long history of use in Japan as ascaricides [107]. The human domoic acid poisoning incident at Prince Edward Island, Canada, in 1987 led to the discovery that DA was produced by microalgae. The main implication of that finding from a public health perspective is that microalgae are mobile and also available to a broader range of marine food products such as planktivorous fish and filter-feeding bivalves, so domoic acid can be widely distributed in marine food webs. Could kainic acid also be produced by an as-yet undiscovered marine diatom, with corresponding public health significance? This supposition may be unlikely, as two decades of natural products research have not found KA being produced by *Pseudo-nitzschia* spp.

Routine food safety screening for domoic acid will not identify kainic acid in marine food products. The ELISA for domoic acid reportedly does not cross-react with kainic acid [108]. However, a more recent paper would appear to contradict this suggestion, with the domoic acid ELISA apparently as sensitive to KA as it is to DA, so the ELISA would overestimate DA when KA is present [109].

KA is produced by several species of red algae (Rhodophyta) [110,111]. The widely distributed and abundant *Caloglossa leprieurii* is an edible seaweed [112], as is *Palmaria palmata*, which is marketed as dulse [18,113]. *Digenea simplex* and *Vidalia obtusiloba* were amongst various seaweeds recently investigated by de Oliveira et al. [114] for their nutritional capacity; interestingly, pooled seaweeds in that study were examined for various toxic and antinutritive factors such as lectins, trypsin and amylase inhibitors and toxic metals, but production of kainate exctitotoxins was not considered. *D. simplex* and *V. obtusiloba* are capable of producing both domoic and kainic acids [110]. Consumption of seaweeds has long been popular in Pacific Island and Pacific Rim countries, and there is a growing aquaculture industry that supplies a growing worldwide demand for edible macroalgae. A 2003 FAO report estimated a global annual seaweed harvest of 8 million wet tonnes and a US$5 billion industry supplying seaweed products for human consumption [115]. Public health research investigations into contaminants of edible macroalgae largely focus on toxic metals such as arsenic and mercury, and radioisotopes [116,117]. There are apparently no monitoring programs or regulatory guidelines for the presence of kainic and/or domoic acids in rhodophyte macroalgae; no food safety research studies to screen edible rhodophytes for these natural toxins have been published.

The question of whether kainic acid can move into marine food webs from rhodophyte macroalgae seems to have received scant attention. Herbivorous fish and crabs would be obvious targets, and might be able to be investigated experimentally in this regard. "Large amounts" of domoic acid detected in the macroalga *Chondria armata* from southern Japan were thought to represent the source of DA detected in the xanthid crab *Atergatis floridus* from that region; those crabs reportedly feed on seaweed [48]. Equivalent pathways to facilitate the entry of kainic acid into certain marine food products might therefore be suspected.

The sensitivity of the developing brain to kainic acid neurotoxicity is somewhat difficult to interpret. Several papers describe a decreased sensitivity in younger laboratory rodents, e.g. [118-120]. Kesslak et al. [121] found the opposite: older rats had less severe KA-induced hippocampal lesions than young animals. Nevertheless, those studies [118-121] investigated different age classes of adult animals, so presumably cannot be applied to the understanding of kainic acid exposure during gestational or neonatal brain development. The abstract of a Japanese-language paper describes behavioural and cognitive impairment in juvenile rats exposed in mid-gestation to a single low dose of KA; an apparently decreased threshold for KA-induced "wet-dog shakes" was also noted [122]. The wet-dog shakes sign is an aberrant

(though not pathognomonic) response seen in laboratory rodents following kainate exposure, and thought to be associated with limbic seizures [123]. A study comparing juvenile, pubescent and adult rats dosed i.p. with kainic acid demonstrated more severe seizures, lower convulsive thresholds and highest mortality in the pups. More severe brain lesions were seen in adult and pubescent animals, but not in pups [124]. This finding of seizure susceptibility but decreased lesional damage in the immature brain compared to the adult brain in response to kainic acid exposure has been repeated in other studies and is discussed in the literature, e.g. [125-127]. Dose–response data from some studies suggests an inverse relationship between age and sensitivity to kainic acid: 1–2 mg/kg doses were used to generate electrographic and behavioural seizures in neonatal rats, 2–10 mg/kg for juveniles, 10–11 mg/kg in pubescents and 10–12 mg/kg in adults (i.p or s.c. route) [125,127,128].

The question of whether and to what degree the human foetal brain may be susceptible to the neurotoxic effects of kainic acid via maternal dietary intake would appear to be an open question. Direct consumption of kainic acid through contaminated edible rhodophyte macroalgae is not monitored from a food safety perspective; trophic transfer of kainic acid to herbivorous fish or crustaceans is conceivable but, again, poorly researched.

Should other harmful algal and cyanobacterial toxins be considered in the search for environmental risk factors for TLE-HS in early development?

This discussion reflects my interest in harmful algal toxins; no particular claim is made that these diverse group of environmental toxicants are inherently more worthy of investigation in this regard than other exogenous agents or endogenous factors that may impair developmental function of the hippocampus.

Marine and freshwater microalgae and cyanobacteria produce a broad array of structurally and mechanistically diverse toxins. One feature common to most is that they are extremely potent, more so than for domoic acid, the principal focus of this paper. Many HAB toxins have LD_{50}s in the sub-ppm range; Pacific ciguatoxin-1 has an i.p. mouse LD_{50} of 0.25 ppb [129]. Therefore many of these compounds are biologically active at extremely low concentrations. Some are water soluble molecules, e.g. domoic acid, saxitoxins, most of the freshwater cyanotoxins. Some are very lipophilic, particularly the cyclic polyether brevetoxins and ciguatoxins. Several algal neurotoxins are potent ion channel modulators. Channelopathies are increasingly the focus of attention by epilepsy researchers [130], and ion channels are seen as a common target for both genetic and non-genetic (i.e. environmental) epilepsy risk factors [131,132]. Some examples of HAB toxins that are of interest to neuroscientists and may also arouse the curiosity of epilepsy researchers:

BMAA

β-N-methylamino-L-alanine (BMAA) is a non-protein excitotoxic amino acid currently being investigated, though not without considerable controversy, in the context of some chronic degenerative neurological diseases including amyotrophic lateral sclerosis and Alzheimer disease. BMAA is undoubtedly neurotoxic, apparently with a preferential affinity for glutamate receptors, though with discrepant reports regarding which receptor sub-types are targeted [133]. Intracranial injection of BMAA in mice has demonstrated preferential acute neurotoxicity of the hippocampus, specifically the CA1 zone [134]. A recent study [135] describes exposing separate groups of mice during prenatal and neonatal periods to tritiated BMAA. Radioactivity was higher in foetal tissues than in maternal blood, which in turn was higher than in maternal brain, indicating an intact blood–brain barrier effect in the adult. There was a gradual selective uptake of radioactive label in the foetal CNS; companion autoradiographic studies in neonatal mice showed preferential uptake into the hippocampus, striatum, thalamus, cerebellum, brainstem and spinal cord. The authors also describe chronic cognitive impairment in neonatal rats dosed with BMAA [135]. In a neurodevelopmental

study using a zebrafish (*Danio rerio*) embryo model, Purdie et al. [136] reported clonic-like convulsions and other abnormalities in embryos exposed to BMAA. Cycads have long been known to produce BMAA, with purported human exposures in localised populations that consume cycad flour. Recent reports suggest that this toxin is produced by a wide range of freshwater and marine cyanobacteria, with implications for much more diffuse entry into the food chain and water supplies [137,138]. Again, this topic is the subject of active and current controversy, with interpretation of the epidemiology and important aspects of the bioavailability and mechanisms of toxicity for the role of BMAA in chronic neurological disease as well as the analytical determination of BMAA in cyanobacteria under dispute [139-142].

Microcystins

These are a family of cyclic heptapeptides produced by freshwater and (possibly) marine cyanobacteria; they are potent hepatotoxins and tumour promoters. Cyanobacteriologists have long recognised these compounds as hepatotoxins, utilising an active carrier mechanism into the liver, and the main toxicological research effort to date has focused on those mechanistic aspects of the microcystins [143]. However, a study from over 20 years ago first raised suspicions that the developing central nervous system may be at risk. Falconer et al. [144] reported that chronic exposure to a microcystin-producing cyanobacterial extract in pregnant mice resulted in reduced brain size in 10% of pups; histological examination showed that the hippocampus was "extensively damaged." An expert group has called for neurodevelopmental toxicity studies to be conducted on all the main groups of cyanotoxins, as this topic is essentially unexplored [145].

An organic anion transporter protein expressed in endothelial cells of the blood–brain barrier has subsequently been shown to bind microcystin, with the implication that this mechanism will allow these cyanotoxins to access the CNS in addition to the known affinity for the liver [146]. A recent report [147] describes intra-hippocampal injection into adult rats of a microcystin-producing cyanobacterial extract. Controls were injected with physiological saline. A series of behavioural tests was used to assess short-term and long-term memory. The authors report some statistically significant outcomes, though no dose–response effect is apparent. The study design can also be criticised for employing cyanobacterial extracts (i.e. impure materials) without related controls for this work involving intra-hippocampal injection [147].

Ciguatoxins

The ciguatoxins are a group of heat-stable cyclic polyether compounds responsible for the ichthyosarcotoxaemia known as ciguatera poisoning. These are highly lipophilic and very potent toxins, causing illness and death in humans who have eaten affected fish containing ciguatoxins at sub- and low-ppb concentrations [129,148]. Ciguatera poisoning is a channelopathy; the toxin acts on site 5 of neuronal voltage-gated sodium channels. Exposure of various types of neurones to nanomolar or picomolar concentrations of ciguatoxins causes a hyperpolarisation that results in sodium channel opening at resting potentials. Ciguatoxins also appear to be K^+ channel modulators [149]. The disease is characterised by a range of debilitating sensory abnormalities that can persist for weeks, months or years after single exposures. Ciguatera poisoning is the most common ichthyosarcotoxaemia, with estimates of up to 500,000 cases annually [150]. Once restricted to tropical regions, where the source microalgae are associated with reef systems, ciguatera is now seen in higher latitudes because of the extensive global export trade in fish and the rapid movement of tourists to and from ciguatera-prone regions.

From a neurodevelopmental perspective, ciguatoxins appear able to cross the placenta. Several case reports describe increased ("alarming" in one case) foetal movement reported by pregnant women; these occurred within hours of consumption of ciguatoxic fish. Ciguatoxins can also be transmitted to infants via breastmilk [151].

Saxitoxins

The saxitoxins are a group of water-soluble tricyclic guanidinium alkaloids, produced by both eukaryotic marine microalgae and freshwater (and possibly marine) cyanobacteria [143,152]. Symbiotic bacteria also appear capable of synthesising saxitoxins [153]. Saxitoxin poisoning occurs via two distinct natural exposure pathways. Contamination of drinking water supplies by saxitoxin-producing freshwater cyanobacteria has caused mass mortalities in stock animals, the most notorious event recorded in Australia in 1991 [154]. More widespread and frequent human intoxications occur from consumption of saxitoxin-contaminated shellfish, crabs and puffer fish [152]. The saxitoxins act at site 1 of voltage-gated sodium channels, blocking the influx of Na^+ into motor neurones, thus causing a conduction block that manifests as respiratory paralysis. Hypoxia and death can follow severe intoxications [143]. Tetrodotoxin is a toxin associated with a variety of terrestrial and marine animals; humans are exposed primarily through consumption of teleost fish from the order Tetraodontidae (pufferfish). Tetrodotoxin is produced by symbiotic heterotrophic bacteria; it is structurally related to saxitoxin, and is functionally identical, also binding to site 1 of voltage-gated sodium channels and causing morbidity and death by acute respiratory failure [153]. Just as the marine algal toxins kainic acid and domoic acid are widely used by neuroscience researchers as potent excitotoxic glutamate analogues, so saxitoxin and tetrodotoxin have similarly broad use amongst experimental neuroscientists as sodium channel blockers. Particular use is made of saxitoxin and tetrodotoxin binding assays to investigate the function of voltage-gated sodium channels (VGSCs). That these algal and bacterial toxins are adopted as the most useful tools for investigating their respective neurological activities is another indication of their extreme chemical potency in biological systems.

I am not aware of either case reports of pregnant women suffering saxitoxin or tetrodotoxin poisoning or experimental work to investigate gestational exposure to these toxins. At first glance their highly polar nature might suggest that they would not cross the placenta very efficiently, but this remains to be tested and at least in this rather narrow context of HAB toxins, other low molecular weight polar molecules can cross the placenta in concentrations sufficient to damage the developing CNS, the most obvious example being domoic acid, the principal topic of this hypothesis.

Other HAB toxins that may be of interest to epileptologists

Spirolides are a family of potent macrolide neurotoxins discovered in 1991 in bivalve molluscs. Parenteral administration of sub- to low-ppm doses to rodents resulted in rapid onset of neurological signs, including generalised "tremors … resembling a seizure" and death within 8 min. Histopathological examination showed the brain was the sole organ affected, with the hippocampus the target structure; CA3 and dentate gyrus were most affected [155]. The okadaic acids are a group of polyether toxins; natural exposures occur from consumption of contaminated shellfish. They are potent protein phosphatase inhibitors. Intra-hippocampal injection of okadaic acid at low micromolar concentrations in rats is followed by acute epileptiform EEG discharges, seizures and neuronal destruction of the hippocampus in zones CA1, CA3 and the dentate gyrus [156]. Palytoxin is a giant polyol that causes an extremely potent channelopathy by binding to Na^+ K^+-ATPase and converting the pump into a non-specific ion channel for monovalent cations. The i.v. LD_{50} in the rabbit is 25 ng/kg, with an approximately equivalent toxicity in dogs. Fatal human intoxications have been reported following consumption of crabs, sea urchins and fish [157-159]. Absorption of the toxin via the dermal route in aquarium hobbyists is reportedly associated with exposure to zoanthid corals [157,160].

The neurotoxins discussed above are a subset of the suite of neurally-active compounds produced by cyanobacteria and marine microalgae. There are many other examples of potent toxins to which humans may be exposed via water and foods of aquatic and marine origin (e.g. anatoxin-a(s), an anticholinesterase; anatoxin-a, a potent acetylcholine agonist, causes a

post-synaptic neuromuscular block; kalkitoxin and antillatoxins, lipopeptides that are potent sodium channel activators and blockers, respectively), and lipophilic toxins that may contaminate shellfish, seafood and edible macroalgae (e.g. polycavernosides, a group of macrolide toxins; symbiodinolide, a polyol macrolide and Ca^{2+} channel activator) [161-164]. A relationship between algal toxins and ion channel function in higher vertebrates may be more fundamental than a cursory examination of the topic might invite. Some workers have suggested that algal toxins may have influenced the evolutionary development of VGSCs, as such toxins constitute offensive and defensive strategies and thus confer a selective advantage on the organism that produces them. These predator/prey interactions are likely to have occurred in ancient marine systems over 700 million years ago, when the evolutionary development of sodium channels began [165]. The extreme potency of these marine toxins may be explained by the observation that the chemical needs to overcome dilution effects of seawater when advancing towards its target [166]. Similarly intriguing aspects of glutamate ligand/receptor interactions, and the actions of the kainate excitotoxins that are the principal focus of this hypothesis, may have their origins in a distant evolutionary past. Glutamate receptor gene sequences have been discovered in plants; while the function of glutamate receptors in plants is not completely understood, they may participate in cell signalling processes. Cell-to-cell signalling by excitatory amino acids in animal neural systems appears to have arisen from ancient signalling mechanisms that arose before the divergence of plants and animals [167,168]. This may explain why plant and algal-derived compounds such as the kainate excitotoxins, as well as other neurally-active ligands such as cocaine, nicotine and caffeine, act on receptors in animal brains [168]. Competitive advantages conferred by allelochemistry may, over evolutionary timescales, have led to the transformation of complex species-specific signalling molecules into toxins in some circumstances.

The toxicology and epidemiology of many algal biotoxins on adult humans is poorly understood, and knowledge of how low dose exposures impact on the developing central nervous system is essentially non-existent. Yet the candidate environmental neurotoxin in this group that deserves scrutiny from the perspective of early developmental insults and TLE-HS should be domoic acid, the premise of this paper. Enough is known about its mechanism of toxicity as an excitotoxic glutamate agonist, its ability to cross the placenta, its heightened potency on the foetal and neonatal brain, its capacity to initiate chronic temporal lobe epilepsy both experimentally and through natural exposures, and its widespread distribution in marine food webs to elevate this toxin to priority status for investigation.

Are mammals more at risk of persistent neurological injury from exposure to kainate excitotoxins because of their hippocampal formation and function?

The only mammals thus far known to be at risk of sub-acute and chronic neurotoxic injury from natural exposures to domoic acid are humans and sea lions; exposures have been documented in various cetacean species, though confirmatory evidence for harmful effects in whales and dolphins has not as yet been reported [169]. While the long-term memory function of humans as a species is well understood, sea lions also have exceptional long-term conceptual memory [170]. The literature on experimental domoic acid neurointoxication using rodent models, as discussed above, demonstrates that chronic cognitive dysfunction and epileptic seizures can be initiated by single exposures in these small mammals. By contrast, experiments on juvenile leopard sharks (*Triakis semifasciata*) exposed to high parenteral doses of DA failed to demonstrate either acute or sub-acute effects after being observed for 7 days [171]. The authors of that study showed that leopard shark brain (principally in the cerebellum) possesses high kainate receptor binding capacities and suggest that one explanation for the absence of toxic effects may be the presence of an endogenous ligand that competitively binds kainate receptors [171]. Northern anchovy (*Engraulis mordax*), the vector for trophic transfer of domoic acid to California sea lions in mass poisonings, have

been shown to exhibit acute neurotoxic behavioural signs following parenteral exposure to DA, though not after gavage experiments [172].

There is clearly a need for research investigation into sub-acute and chronic effects of discrete exposures to domoic acid within and across different taxonomic groups that are associated with marine food webs. Observational studies in wild animals are very helpful but limited in their ability to relate chronic adverse neurological outcomes to known patterns of exposure to domoic acid. Are observed chronic effects due to earlier single exposures, or could chronic exposures be occurring? Dose–response curves, toxicokinetics studies and measurement of chronic endpoints after exposure to DA by natural exposure routes would be very valuable experimental contributions. Experiments could be designed for representative species of fish, birds, cephalopods and other marine invertebrates to investigate the chronic neurotoxicity of domoic acid. Similar experiments with pinnipeds and cetaceans would present considerable ethical hurdles, and it may be difficult to identify control animals with a known absence of dietary exposure to domoic acid. Neuroimaging studies involving post-mortem MRI acquisition of cetacean brain structures may help to answer some of these questions. Cetacean brain MRI studies have been conducted and a growing database will assist in providing reference images for comparison with suspected domoic acid intoxications in these marine mammals [173,174]. Such a database would be particularly useful if it could include findings from whales and dolphins known to have been unexposed to DA, including through gestation, though it is difficult to envisage how such animals could be identified other than in captive animals, and then only if their food sources had been screened for domoic acid – not a trivial exercise. But the question heading this section may be answerable in part by investigating the capacity for domoic acid to initiate sub-acute and chronic injury across various animal groups. Is there something fundamental and important about the mammalian hippocampus that renders them more vulnerable to the toxic effects of excitatory neurotoxins? Or are findings of chronic effects only in humans and sea lions following natural exposure to DA merely incidental and simply an indication that this whole topic is so poorly understood and under-researched? Could studies in comparative anatomy and physiology of the hippocampus across various species help to answer this question? Is it feasible that humans and pinnipeds are more susceptible to acute, sub-chronic and chronic cognitive dysfunction caused by exposure to domoic acid because of similarities in hippocampal cellular organisation and architecture, compared to (say) fish and birds? Pelicans that survived an apparent DA poisoning incident were reportedly showing signs of disorientation and weakness 2 months after the event [175], though again the issue of whether chronic effects from acute exposure or continuing acute effects of chronic exposure were operating remains unanswered, and may be difficult to answer observationally. Birds have homologous structures to the mammalian hippocampus, though the gross architecture and laminar structure of the avian hippocampus is different to that in mammals in several respects. The hippocampus in birds is located in more medial, superficial and superior aspects of the brain, and distinct morphological features such as the dentate gyrus, *Cornu ammonis*, and hilar region are absent. However, the avian hippocampus is described as having comparable functional properties to that of the mammalian structure; many bird species, in particular migratory and food-storing types, have highly-developed long-term memory functions, and the hippocampus appears to be fundamentally involved in this capability [176,177]. Fish do not have a brain structure that is comparable to the mammalian hippocampus, though some fish appear capable of long-term memory function; there are intriguing anecdotal reports of catfish with a five-year memory capacity [178].

As to why mass strandings occur in toothed whales and dolphins but not in baleen whales (suborder Mysticeti), could exposure to domoic acid be offered as a partial explanation? The most frequent supposition here is that the highly developed social behaviour of odontocetes explains this phenomenon. As the majority of animals in a stranded pod are apparently healthy, social behaviour has been posited to explain this behaviour. So-called conformist culture is thought to influence these events, where presumably physiologically normal

animals essentially commit mass suicide due to social imperatives to remain with the group [179]. But are all these animals healthy? How would disorientation and confusion resulting from group dietary exposure to an environmental neurotoxin in localised prey manifest in immobilised whales and dolphins? Would such effects, if present, be observable by field investigators? Lining up against the domoic acid hypothesis in this regard would be the supposition that severe acute intoxication by domoic acid might be expected to cause convulsive seizures, perhaps even *status epilepticus* in a proportion of beached pod members, and these would presumably be noted by competent veterinary field workers. This assumes that electrical seizures or *status epilepticus* in stranded cetaceans that might be caused by exposure to domoic acid would present with obvious clonic movements, and if that were to be the case that such events would not then be interpreted as part of a pre-moribund condition resulting from respiratory insufficiency and hypoxia.

The cetacean hippocampus is remarkably underdeveloped, given the robust capacity for learning and long-term memory in this group; the hippocampus in a representative mysticete "further diminutive" than in odontocetes [180]. Some workers suggest that these functions may have transferred to the adjacent and well-developed cortical limbic lobe (entorhinal cortex and periarchicortex above the corpus callosum). Marino [181] suggests that "only odontocetes show a possible compensatory development in the form of a highly convoluted and massive limbic lobe" though it is unclear whether or not that statement, made by the author in comparing brain development across cetaceans, pinnipeds and sirenians, also invites comparison between odontocete and mysticete brain structure. If domoic acid is causing neurointoxication in cetaceans, could differences in stranding behaviour between odontocetes and mysticetes be explained by differential brain pathologies, or could dissimilarities in the feeding ecology within and between these groups offer a more plausible account? The Balaenid whales, comprising the right whales, bowhead whale (*Balaena mysticetus*), gray whale (*Eschrichtius robustus*) and the pygmy right whale (*Caperea marginata*), consume small crustacea. The other representatives of the baleen whales, the rorqual family, feed on krill but also hunt schools of herring, pilchards and sprat. Could feeding at different trophic levels, bioaccumulation of DA in planktiovorous schooling prey fish of odontocetes and rorquals, and differences in social feeding behaviour point to dietary influences for some mass strandings?

Concluding remarks

The developing hippocampus would seem to be exquisitely sensitive to a disparate array of environmental risk factors capable of initiating long-lasting injury to specific cellular zones. Prenatal exposure to ethanol, stress (modelled experimentally by maternal restraint), protein malnutrition, nicotine, opiates and various xenobiotics and pharmaceuticals have all been shown to cause discrete pathological changes in the developing hippocampus [182-188]. Excitotoxic neural injury in the developing brain may be associated with various insults, including hypoxic/ischaemic events or vascular insufficiency [189].

Unknown environmental risk factors in the early developmental period presumably operate to explain a significant proportion of TLE cases that can be categorised as cryptogenic TLE and TLE with hippocampal sclerosis. The environmental insults that come into play here may well be heterogeneous. But the premise of this paper is that domoic acid represents an important candidate environmental neurotoxin that should be given priority status for research investigation into gestational environmental risk factors for temporal lobe epilepsy of unknown aetiology. It is a known excitotoxin, it initiates chronic temporal lobe epilepsy after single exposures in experimental animals and natural poisonings in humans and wild animals. It crosses the placenta, causes unilateral and bilateral hippocampal pathology as well as extra-hippocampal damage by natural exposure routes. The developing brain appears to be significantly more sensitive than the mature brain to the neurotoxic effects of DA. And it is

present in marine food webs, though this aspect is currently much under-researched. If gestational exposure to domoic acid only explains a small proportion of the attributable risk for TLE of unknown aetiology, the public health benefit from investigating, characterising and quantifying this particular environmental exposure would still be very significant, as this would represent a potentially preventable exposure. Public health advisories recommend pregnant women avoid or limit consumption of specific fish and whale meat in order to limit their exposure to methylmercury [190,191], the toxic effects of which on the developing central nervous system are well understood. If prenatal exposure to domoic acid is found to explain even a small proportion of TLE cases, the ability to prevent a lifelong, frequently relapsing and costly chronic disease by advising pregnant women to avoid consumption of specific marine foods at critical periods of their pregnancy would be an appropriate public health intervention.

Engel et al. [192] highlight the difficulty of considering early developmental precipitating factors for epilepsy, where the disease may not reveal itself until many years have passed:

> "The lesson of epilepsy has been that the faults are often … prenatal … or early postnatal, but the … epilepsy … becomes "eloquent" at some later stage … The developmental perspective is to consider the possibility that the lesion or fault could have been "eloquent" in other ways before the condition of interest arose…This removes a need for thinking that a chronic lesion that arose in embryonal life has somehow acquired new powers at age 14 (or whenever) because it has started to cause epilepsy." [192].

The foetal brain is much more susceptible to a wide variety of toxic insults than the adult brain. A "silent pandemic" of neurodevelopmental disorders reportedly operates from exposures *in utero* to various inorganic and anthropogenic chemicals; silent in the sense that the effects of many such exposures are unrecognised and therefore unreported [193]. Low dose exposures to domoic acid may also potentially place the foetal brain at risk, and such exposures may occur without recognition by mothers and health workers, i.e. they may also be epidemiologically silent. Banerjee and Hauser, in concluding their chapter on the incidence and prevalence of epilepsy, suggest that a fundamental objective predicated on an enhanced understanding of the epidemiology of epilepsy should be prevention of the disease, and that such gains in understanding might be achieved in part by asking "more pointed questions" [70]. Collecting exposure data on dietary seafood intake during pregnancy from mothers of cases and reference subjects recruited into future prospective and retrospective investigations would be an appropriate and low-effort approach to begin investigating the potential for this seafood-borne excitotoxin to function as a risk factor for TLE-HS.

Domoic acid, and its macroalgal analogue kainic acid, are widely used as chemical models to investigate various excitatory neurological processes and pathologies, including limbic epilepsy. These compounds should also be viewed by neuropathology and epilepsy researchers from another direction, not just as tools but as toxins, and more specifically as environmental neurotoxins. Such endeavours will address knowledge gaps in the neurotoxicity, food chain dynamics, toxicokinetics and eventually the environmental epidemiology of the kainate excitotoxins.

Conflict of interest statement

None to declare

Acknowledgements

This work was funded in part by the Queensland Government Cabinet-approved QHFSS research fund, project #RSS08-005. Thanks to Gustaaf Hallegraeff and Sue Poole for constructive feedback on an earlier draft of the paper.

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