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NEUROLOGICAL IMPACTS OF PROLONGED ASYMPTOMATIC EXPOSURE TO THE MARINE NEUROTOXIN DOMOIC ACID

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by

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NEUROLOGICAL IMPACTS OF PROLONGED ASYMPTOMATIC EXPOSURE TO THE MARINE NEUROTOXIN DOMOIC ACID

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ABSTRACT

Domoic acid is an algal-derived neurotoxin that contaminates seafood during harmful algal blooms. It causes excitotoxicity in the vertebrate central nervous system by over-stimulating neurons; in high doses, this can cause seizures, brain lesions, and death. At low doses, however – those causing no outward signs of excitotoxicity – it is unclear whether domoic acid exposure could have potentially damaging neurological effects. This is especially relevant for human exposures, as we are protected from high toxin levels by seafood monitoring and regulatory efforts, but can still ingest domoic acid at concentrations below the regulatory limit (20 µg domoic acid per g wet shellfish weight). To address whether chronic exposure to these low-levels of domoic acid negatively affects neurological health and function, I: 1) examined changes in gene expression and cellular energetics in the brains of adult zebrafish chronically exposed to asymptomatic domoic acid; 2) assessed the effect of low-dose (i.e., non-cytotoxic) domoic acid exposure on the electrophysiological activity and connectivity of neural networks in organotypic mouse brain slice cultures *in vitro*; and 3) examined the brains of adult mice chronically exposed to asymptomatic domoic acid for signs of histopathology and disruption to a subtype of parvalbumin-positive inhibitory neurons. I found that

prolonged domoic acid exposure significantly affected gene transcription and impaired mitochondrial function in zebrafish brains, and altered neuronal network activity and connectivity in mouse brain slice cultures, all in the absence of overt symptoms, gross histopathology, and neuron death/injury. Further, chronic asymptomatic domoic acid exposures that led to transient deficits in learning and memory in a mouse model did not produce measurable effects on hippocampal inflammation, cell/neuron numbers, or parvalbumin-positive staining intensity. These results indicate that chronic domoic acid exposure may have sub-clinical effects that can go undetected and may impact neurological function; further study is needed to better inform human health risk assessments.

This dissertation is dedicated to my wonderfully unique family – I couldn't have accomplished this without you!

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CHAPTER 1 – INTRODUCTION

Background

Phytoplankton – photosynthetic, single-celled algae – are a critical component of marine and global ecosystems: They comprise the base of the marine food web and supply a significant amount of the oxygen in the atmosphere. However, some phytoplankton are known to produce harmful toxins, such as domoic acid, during "bloom" events, or periods of intense algal growth. Domoic acid, a neurotoxin produced by marine phytoplankton of the genus *Pseudo-nitzschia*, can permeate the marine food web during harmful algal blooms and poison organisms in upper trophic levels. Such poisoning events can negatively impact wildlife health and fish/shellfish industries across the globe.

Harmful algal blooms

The definition of "harmful algal bloom" is not clear-cut, as the term is societal rather than scientific; in general, a harmful algal bloom event is *any aquatic bloom of microscopic or macroscopic growth that has a detrimental effect on wildlife, human health, and/or national economies*. Algal blooms require a combination of favorable nutrient, temperature, and light conditions, meaning that they are closely tied to seasonal cycles and nutrient availability. Blooms most often occur in coastal regions, which are naturally productive due to upwelling of nutrient-rich deep ocean waters. Anthropogenic factors, like agricultural run-off, can also increase nutrient availability and promote bloom formation (Anderson et al., 2002).

Blooms of certain algae types can wreak havoc on the marine ecosystem, with negative impacts usually manifesting in one of three ways: 1) mass mortality of

marine fauna through decomposition-induced oxygen depletion (Diaz and Rosenberg, 2008); 2) selective wildlife kills by production of hydrogen peroxide or other reactive oxygen species (Oda et al., 1992; Yamasaki et al., 2004); or 3) toxin vectoring. Toxin vectoring occurs when phytoplankton-produced toxins (phycotoxins) concentrate in primary consumers during harmful algal bloom events, leading to significant transfer to secondary consumers. Examples of phycotoxins include saxitoxin and domoic acid, which are responsible for Paralytic Shellfish Poisoning and Amnesic Shellfish Poisoning, respectively.

Domoic acid as a phycotoxin

Domoic acid is a water-soluble, polar, non-protein amino acid (311.3 g/mol) produced by phytoplankton of the diatom genus *Pseudo-nitzschia* (Subba Rao et al., 1988). *Pseudo-nitzschia* are found in oceans across the globe, and although not all species produce domoic acid, the toxin has been measured in shellfish, fish, and crustacean samples around the world (Amzil et al., 2001; Blanco et al., 2010, 2002; Costa et al., 2005, 2003; Hess et al., 2001; Lopez-Rivera et al., 2009). The purpose of domoic acid production by *Pseudo-nitzschia* spp. is not entirely clear, but has been linked to nutrient conditions and has been proposed to function as a trace metal-binder (Mos, 2001) or predation-deterrent (Tammilehto et al., 2015).

In cell culture studies, silicate- and phosphate-limitation facilitates toxin production; it is thought that nutrient limitation suppresses primary metabolic pathways and shifts cellular resources to secondary metabolic pathways like domoic acid production (Pan et al., 1998). Nitrogen-limitation inhibits domoic acid production by depleting the free cellular nitrogen required to build nitrogenous compounds (Pan et al., 1998). Other studies have found that domoic acid can chelate both iron and

copper, with proposed roles in nutrient-acquisition and detoxification, respectively (Maldonado et al., 2002; Rue and Bruland, 2001; Wells et al., 2005). Presence of copepods – a type of zooplankton that preys upon phytoplankton – was also shown to induce toxin production in *Pseudo-nitzschia seriata*, though domoic acid's efficacy as a predation defense was not assessed (Tammilehto et al., 2015).

Movement of domoic acid through the food web

Like some other harmful algal bloom toxins, domoic acid is able to move through the food web through concentration in primary consumers. Planktivorous organisms, such as shellfish (clams, mussels, scallops) and filter-feeding finfish (anchovies, sardines, mackerel) accumulate domoic acid (Lefebvre et al., 2002a, 2002b; Wekell et al., 1994) and can transfer high toxin concentrations to upper trophic levels during bloom events. Consuming contaminated seafood can lead to morbidity and mortality in marine mammals, sea birds, and humans (Bates et al., 1989; Perl et al., 1990; Scholin et al., 2000; Sierra-Beltran et al., 1997; Work et al., 1993; Wright et al., 1989), with symptoms ranging from gastrointestinal (GI) distress and headaches to seizures, brain lesions, coma, and death (Perl et al., 1990; Teitelbaum et al., 1990).

The first recognized human exposure event occurred in 1987 in eastern Canada, when 143 people became ill and 4 died following consumption of domoic acid-contaminated blue mussels (*Mytilus edulus*) (Bates et al., 1989; Perl et al., 1990; Wright et al., 1989). The most common persisting symptom in human survivors was permanent short-term memory loss, leading to the name "Amnesic Shellfish Poisoning (ASP)" (Wright et al., 1989). A regulatory limit of 20 µg DomA/g shellfish tissue (wet weight) was rapidly established for the Canadian shellfish industry

(Wekell et al., 2004) and was adopted by the United States shortly afterward. Other countries in Europe, Asia and Australasia have also taken measures to monitor domoic acid in shellfish and assess the risks associated with consumption (Park, 1995; Toyofuku, 2006).

Although human exposure to domoic acid is relatively well-controlled through monitoring programs, exposure still poses a significant threat to wildlife populations and shellfish industries around the world. On numerous occasions, domoic acid poisoning has caused death and illness in hundreds of seabirds and marine mammals (Gulland et al., 2002; Scholin et al., 2000; Sierra-Beltran et al., 1997; Torres de la Riva et al., 2009; Work et al., 1993), most noticeably along North America's Pacific coast. The Wildlife Algal-toxin Research & Response Network (WARRN-West; NOAA, Seattle) monitors phycotoxin presence in marine mammals, and has regularly found domoic acid in varied species ranging from southern California to Alaska (Lefebvre et al., 2016). Of the many species impacted, California sea lions serve as excellent sentinels of domoic acid's influence on marine wildlife and ecosystem health as they are highly visible and well-studied (Bargu et al., 2010; Bejarano et al., 2008; Goldstein et al., 2008).

Domoic acid's chemical and biological properties

Chemical properties of domoic acid

Domoic acid is an amino acid and structural analogue of kainic acid, glutamic acid, and aspartic acid (Fig. 1), and has a charge of -2 at oceanic and physiological pH (~8.14 & ~7.35, respectively) (Ramsdell, 2007). It is stable at room temperature

but degrades at temperatures higher than 50°C and at extremely acidic (pH ≤ 2) or basic (pH ≥ 12) conditions (Quilliam, 2003). It is also very UV-sensitive, and photo-degradation is the primary elimination route for free domoic acid in the water column (Bouillon et al., 2006). However, for unknown reasons, cooking contaminated shellfish samples does not significantly reduce toxin concentration (McCarron and Hess, 2006).

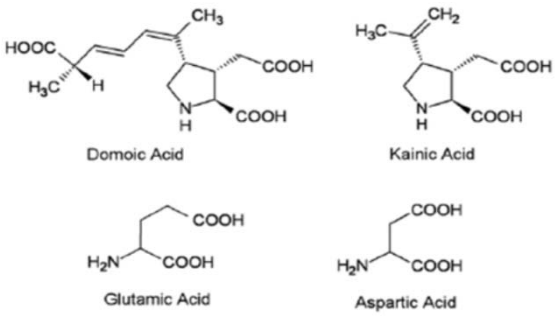

Figure 1. Chemical structure of domoic acid and its biologic analogues (Lefebvre and Robertson, 2010).

Toxicokinetics

Following consumption of contaminated seafood, uptake of domoic acid across the gastrointestinal (GI) tract is low. It has been suggested that GI tract length may affect oral sensitivity to domoic acid. For example, fish and rodents, with comparatively shorter GI tracts, are not visibly affected by oral doses of domoic acid that cause symptoms in primates and humans (Lefebvre et al., 2007). An oral dose of ~1 mg domoic acid / kg body weight causes observable symptoms in humans and primates; by comparison, mice and rats require doses of ~35 mg/kg and ~80 mg/kg, respectively, for observable effects, and fish can receive oral doses of up to 165 mg/kg with no visible symptoms (Lefebvre et al., 2012b).

Once in the bloodstream, domoic acid is rapidly (within 24-48 hours) depurated through the kidneys and urine, with very little metabolism of the compound to its isomers (Suzuki and Hierlihy, 1993). Target organs include the brain, heart (Vranyac-Tramoundanas et al., 2008; Zabka et al., 2009), and kidneys (Funk et al., 2014); neurological symptoms are often the most prominent.

Mode of neurotoxicity

Domoic acid is able to cross the blood brain barrier (via an unknown mechanism) (Preston and Hynie, 1991) and interact with glutamatergic neurons in the central nervous system. Glutamate (Glu) is the primary excitatory neurotransmitter in the brain, acting as a chemical messenger between neurons through ionotropic receptors. Ionotropic receptors serve as ion channels when bound to Glu or another closely-related molecule. Domoic acid, as a structural analogue of glutamate (Fig. 2), is able to bind to ionotropic Glu_Rs (Qiu et al., 2006) with high affinity (Hampson and Manalo, 1998) (Table 1).

The three subtypes of ionotropic Glu_Rs are named for chemicals that bind more selectively than Glu: NMDA (N-methyl-D-aspartate), AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), and KA (kainic acid). When a ligand binds to an ionotropic Glu_R, its pore opens for neuronal depolarization ($AMPA_R$ & KA_R) or calcium influx ($NMDA_R$). Laboratory studies conducted in rodent models have determined that domoic acid binds preferentially to KA_Rs and $AMPA_R$s (Pérez-Gómez et al., 2010).

Table 1. Inhibition constants, dissociation constants, and binding affinities of domoic acid, glutamate, and kainic acid (n*M*) for ionotropic glutamate receptors (AMPA & KA) and their subtypes (where available in the literature).

	AMPA Receptors				KA Receptors				
	GluR1	GluR2	GluR3	GluR4	GluR5	GluR6	GluR7	KA-1	KA-2
Domoic Acid (K_i/IC_{50}) (Hampson and Manalo, 1998)				4640	2	6	37	11	275
Domoic Acid (IC_{50}) (Hampson et al., 1992)	9				5				
Glutamate (IC_{50}) (Hampson and Manalo, 1998)	111	170		1770	290	306	2194	18	480
Kainic Acid (IC_{50}) (Hampson et al., 1992)	3000				7				
Kainic Acid (IC_{50}) (Hampson and Manalo, 1998)	310	220		8650		11		2	
AMPA (binding affinity) (Hunter et al., 1990)	5								
AMPA (IC_{50}) (Hampson et al., 1992)					3600				

When domoic acid binds to Glu_Rs, it activates the receptor and inappropriately depolarizes neurons and/or increases Ca^{2+} influx; at high doses, this contributes to apoptotic and necrotic neuronal death (Ananth et al., 2001; Carvalho Pinto-Silva et al., 2008; Erin and Billingsley, 2004). Necrosis of glutamatergic neurons further propagates excitotoxicity by spilling additional Glu into the extracellular space, over-stimulating nearby neurons, and spreading the damage (Purves et al., 2008). Excitotoxicity leads to increased electrical signaling among

neurons, including seizure activity. Lesions may form in affected brain areas if repair/detoxification mechanisms cannot keep up.

Known neurological effects of DomA exposure

As toxicological outcomes vary with toxin concentration, exposure duration, and number/frequency of exposures, it is important to consider the exposure scenario when examining a compound's toxic effects. *Acute* exposures are usually considered to be one-time or multiple exposures to high toxin concentrations that occur over a short time period (e.g., <30 days), and usually result in severe symptoms. *Chronic* exposures are often considered as repeated, lower-level exposures over an extended period (e.g., >90 days), and that may not elicit overt visible symptoms until later in the exposure regimen, if at all. During chronic exposure scenarios, repeated insults with subtle (e.g., asymptomatic) toxicity may cause cumulative compensatory responses or damage to detoxification mechanisms or organ systems. Cumulative damage may differ from acute effects, making it difficult to infer chronic toxicity from acute symptoms.

Neurotoxicity of acute/high-dose domoic acid exposure

The neurological response to acute domoic acid exposure has been well-characterized in humans, laboratory animal models, and wildlife populations. The most prominent outcome is excitotoxicity, leading to injury and death of glutamatergic neurons in the central nervous system (CNS) through apoptotic and necrotic pathways (Ananth et al., 2001; Carvalho Pinto-Silva et al., 2008; Erin and Billingsley, 2004). Seizures, brain lesions and/or hippocampal atrophy have been characterized in humans (Perl et al., 1990; Teitelbaum et al., 1990), non-human primates (Scallet

et al., 1993), rodents (Ananth et al., 2001; Scallet et al., 2005) and sea lions (Montie et al., 2010; Silvagni et al., 2005) following acute exposure to domoic acid in field and laboratory settings. Laboratory studies have also shown, interestingly, that neurological sensitivity to domoic acid does *not* differ across species as oral susceptibility does. That is, injecting domoic acid directly into the bloodstream or body cavity results in similar EC_{50} values (the dose at which 50% of a population is affected) across fish, rats and mice (2-6 µg DomA/g) (Lefebvre et al., 2001; Strain and Tasker, 1991; L Tryphonas et al., 1990a), though these species differ in the uptake of domoic acid following oral exposure (Iverson et al., 1990; Lefebvre et al., 2007; L. Tryphonas et al., 1990).

The hippocampus, a brain region involved with spatial learning and memory, is highly susceptible to acute domoic acid toxicity as it is especially rich in glutamatergic neurons. Acute domoic acid exposure has also been shown to damage the hippocampus and impair short-term memory and learning (Clayton et al., 1999; Sutherland et al., 1990). Domoic acid-induced neuronal injury and death initiates a glial response (Ananth et al., 2003, 2001; Appel et al., 1997; Mayer et al., 2001; Scallet et al., 1993), in which astrocytes and microglia are activated and recruited to the injury site. Reactive glia interfere with neuron repair and regeneration through the release of pro-inflammatory cytokines, signaling factors and inhibitory molecules (Block et al., 2007; Kim and de Vellis, 2005; Purves et al., 2008). This glial scarring contributes to the formation of lesions at the site of injury.

Neurotoxicity of chronic domoic acid exposure

Attention in the harmful algal bloom research community has recently turned toward elucidating the effects of more prolonged domoic acid exposure, as that

represents the natural exposure scenario for marine wildlife. Marine mammal and seabird populations may be repeatedly exposed to domoic acid through prey items, depending on algal bloom conditions, which results in highly variable doses throughout their lifetime. Understanding the effects of chronic exposures may provide information valuable to monitoring and conservation of endangered marine species, such as the Steller sea lion (*Eumetopias jubatus*), by informing treatment options in rehabilitation agencies and improving exposure risk assessment.

Chronically exposed California sea lions exhibit status epilepticus (Goldstein et al., 2008) characterized by spontaneous, recurring seizures; this syndrome has also been experimentally induced through domoic acid kindling in laboratory rats (Muha and Ramsdell, 2011). Additionally, domoic acid-induced brain lesions in the right hemisphere of the hippocampus in sea lions have been linked to spatial memory deficits, with implications for impaired foraging and increased stranding risk (Cook et al., 2015). While these cases are highly pertinent to wildlife exposures, and could be readily extrapolated to other species with similar exposure risk, they may not be able to inform human exposure particularly well as wildlife exposure history is completely unknown.

Low-dose and developmental exposure to domoic acid

Human exposures will almost always be below the regulatory limit (20 µg domoic acid/g shellfish (wet weight)), and exposure frequency varies with blooms and shellfish consumption frequency. The importance of understanding the difference between high- and low-dose exposures is highlighted by study findings from Lefebvre et al. (2009) showing in zebrafish that brain gene expression response to a single symptomatic dose (1.2 µg domoic acid/g fish) of domoic acid differs from

that of an asymptomatic dose (0.47 µg/g) (Lefebvre et al., 2009): Different domoic acid doses activate different response pathways, suggesting that knowledge of acute dose toxic responses may be insufficient to infer responses to lower-dose toxicity. Chronic low-dose exposure has also been linked to increased neurological sensitivity to domoic acid and production of a domoic acid-specific antibody in zebrafish (Lefebvre et al., 2012a). Currently, these are the only studies that examine neurological consequences of low-level/ asymptomatic domoic acid exposure in the adult brain.

Other domoic acid studies have utilized a postnatal developmental exposure paradigm to take advantage of the fact that a significant portion of neurodevelopment in rats occurs after birth. For example, several research groups based in Prince Edward Island, Canada, have exposed neonatal rats daily to an asymptomatic low dose of domoic acid (20 µg/kg; well below the EC_{50}) during post-natal days 8-14 – a period that coincides with a critical window of hippocampal development. Analysis of the animals' brains as adults (PND 90) showed lasting changes in hippocampal neuronal architecture (increased mossy fiber connections in the CA3 and dentate gyrus of the hippocampus, elevated neurotrophic factor receptor expression in CA3 and dentate gyrus (Bernard et al., 2007), and regional and sex-dependent changes in specific subpopulations of inhibitory interneurons in the hippocampus (Gill et al., 2010)) and altered behavior, memory function, social interaction, and stress response (Adams et al., 2009; Doucette et al., 2004; Gill et al., 2012; Marriott et al., 2012; Ryan et al., 2011). While these studies provide valuable information on the lasting consequences of low-level/asymptomatic developmental domoic acid

exposures, the results may not translate well to chronic, low-dose exposure in the adult brain.

Broader implications and research questions

Changing exposure risks

Harmful algal bloom range and frequency has increased with global climate change. Increases in sea surface temperature have expanded favorable phytoplankton habitats over the past few decades, and harmful algal bloom events are occurring more often, lasting longer, and over greater area (Figs 2 & 3) (Anderson et al., 2012; Fryxell et al., 1997; Hallegraeff, 1993; Jester et al., 2009; Moore et al., 2008; Van Dolah, 2000). Last summer, a warm-water anomaly off the Pacific coast of North America triggered a phytoplankton bloom of unprecedented size and duration (NOAA, 2015). With harmful algal bloom occurrence on the rise, wildlife and human populations are predicted to face increased exposure to algal toxins and the accompanying health risks.

Although the impacts of domoic acid exposure on marine wildlife health have been well-documented, human exposure risks are historically understudied. While humans may be at little or no risk for high-dose domoic acid exposure due to monitoring and regulation of commercial and recreational seafood harvests, the frequency of low-level domoic acid contamination in seafood will likely increase (Lefebvre and Robertson, 2010). People who consume shellfish multiple times per week may be especially at risk, including tribal populations in the Pacific Northwest, regular seafood consumers, and commercial fishermen sampling their own catches. The potential health impacts of chronic low-level/asymptomatic exposure have yet to

be characterized in laboratory models or human populations, leaving many questions unanswered.

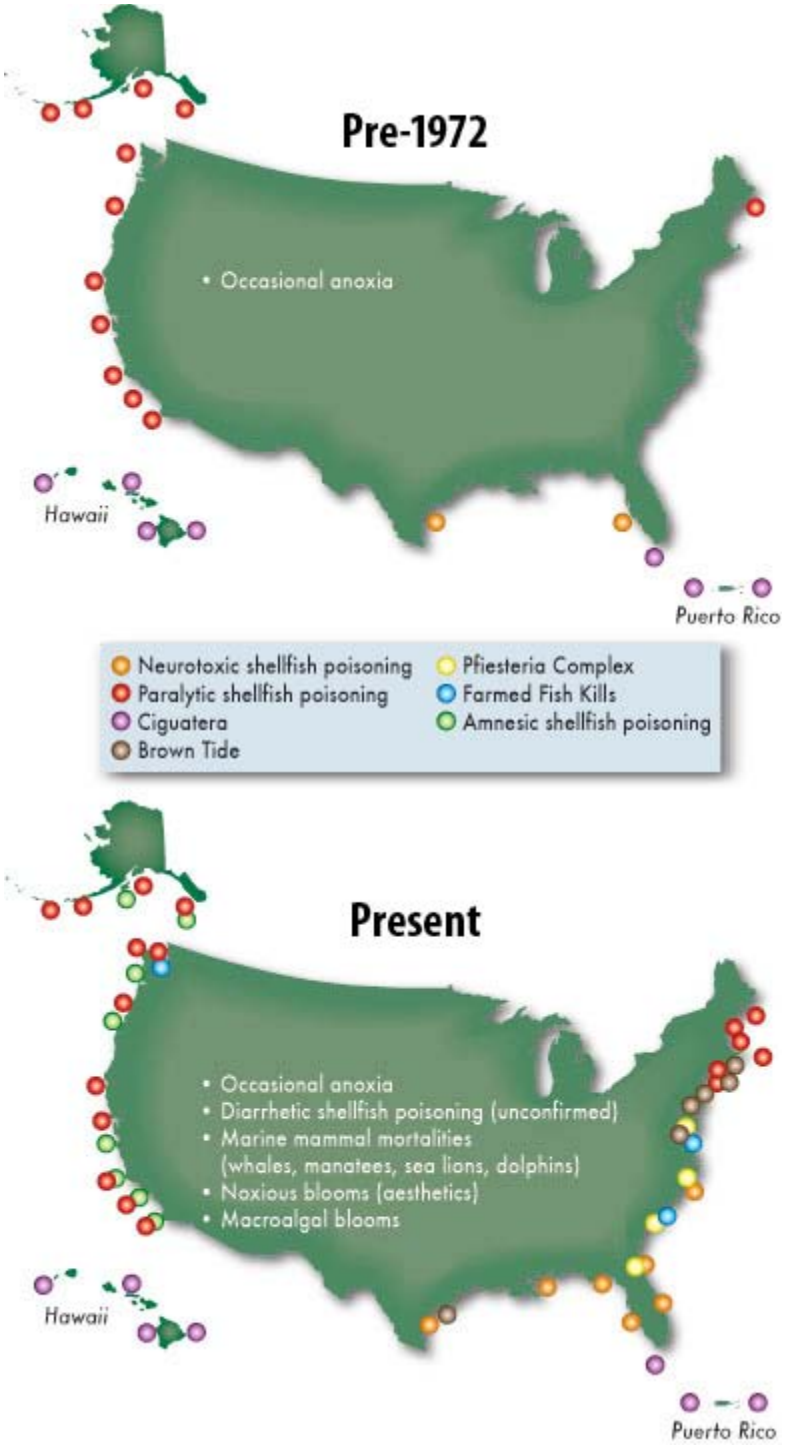

Figure 2. Incidence of harmful algal blooms (Amnesiac Shellfish Poisoning = domoic acid; in green) in U.S. waters pre-1972 (top) and post-1972 (bottom). (credit to: Don Anderson and Jayne Doucette, Woods Hole Oceanographic Institute Graphic Services.)

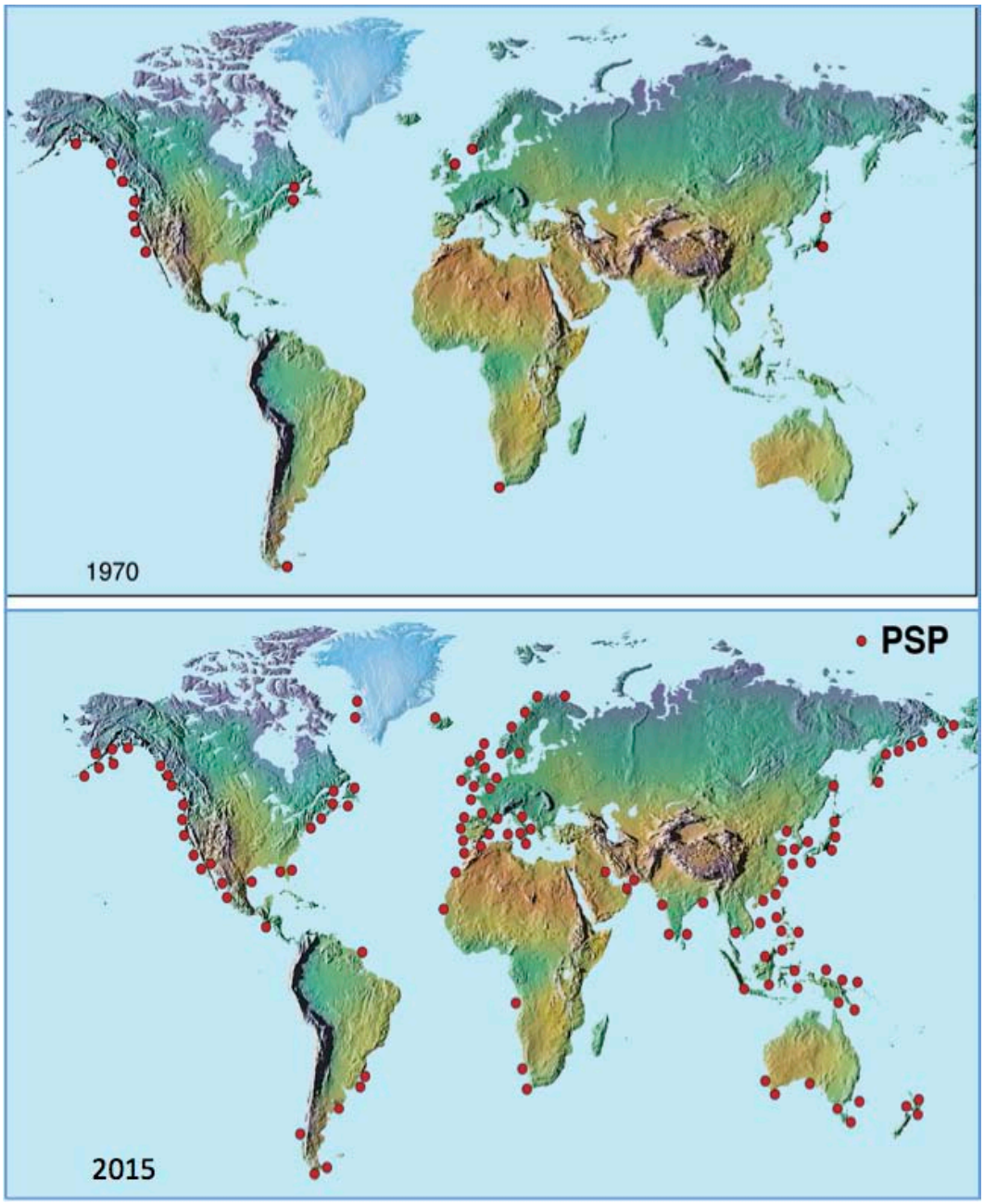

Figure 3. Global incidence of paralytic shellfish poison (PSP toxins) in fish and shellfish samples leading up to 1970 (a) and 2015 (b). (Woods Hole Oceanographic Institute)

Research questions

My research aims to further our understanding of whether chronic exposure to low-dose/asymptomatic domoic acid poses unknown health risks to humans. I began by quantifying cellular-level responses to chronic asymptomatic domoic acid exposure, specifically gene expression changes and disruption of mitochondrial function in the brain. I then expanded my focus to neuron-neuron interactions and how prolonged domoic acid exposure at non-cytotoxic doses affects neuron activity and network connectivity. Finally, I conducted a histological assessment of the hippocampus following chronic asymptomatic exposure to link morphological tissue-level effects to transient functional deficits in learning and memory. Through the course of my research, I have uncovered highly specific changes in the brain caused by prolonged low-level domoic acid exposure. My dissertation describes domoic acid-induced changes in gene expression, mitochondrial function, and neuronal network activity and connectivity, all of which occur in the absence of the overt neuronal injury and death commonly reported with acute toxicity.

In my first research chapter (dissertation Chapter 2), we used a zebrafish model to: 1) Characterize the brain's transcriptome response to prolonged low-dose (i.e., asymptomatic) domoic acid exposure, with hopes of identifying a suite of genes – a transcriptional 'biomarker' – characteristic of that exposure paradigm; and 2) Quantify domoic acid-induced changes in mitochondrial function within the brain. Zebrafish are well-established as an excellent model both for genetic studies and for assessment of the neurological impacts of domoic acid exposure. Of particular note, this study used an Agilent microarray platform to assess changes in gene expression in whole brain tissue at six time points (2, 6, 12, 18, 24, and 36 weeks) during bi-

weekly domoic acid exposures; mitochondrial function was assessed at 36 weeks by Western blot and Oxygraph assays.

In my second research chapter (dissertation Chapter 3), we measured the effects of prolonged low-level domoic acid on the maturation of neuronal networks in an organotypic brain slice culture model. Organotypic brain slice cultures maintain cellular composition and organization of corticohippocampal tissue, and provide direct access to hippocampal circuits, especially useful for measuring electrophysiological activity from hundreds of neurons. To determine how domoic acid influenced network activity and connectivity, we cultured organotypic mouse brain slices; half of the slices were exposed to a non-cytotoxic level of domoic acid in the culture medium for 12 days *in vitro*. At the end of the culture period, we recorded electrophysiological activity from these slices with a state-of-the-art 512-electrode array. Sophisticated computational analyses allowed us to identify individual neurons, assess neuronal and network activity patterns, and quantify neuronal connectivity within the network.

Finally, in my third research chapter (dissertation Chapter 4), we investigated links between domoic acid-induced perturbations in the brain and neurological deficits caused by chronic low-dose exposure in an adult mouse model. These experiments aimed to connect some of the dots between subtle histological changes in the brain – e.g., markers of neuronal injury, protein expression changes, etc. – and functional impairments that affect important cognitive processes. Adult mice were injected weekly with a low, asymptomatic dose of domoic acid for 22 weeks, when brains were collected and processed for immunohistochemistry.

Broader impacts

Ultimately, these projects can provide information in answering long-term questions like: Is the regulatory limit protective enough? Should it be changed? What other monitoring and/or regulatory efforts need to be included? Improving our understanding of the links between our oceans and human health can help balance the need to protect livelihoods and cultural traditions along our coasts with the need to protect people from potential health risks.

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CHAPTER 2 – CHRONIC LOW-LEVEL DOMOIC ACID EXPOSURE ALTERS GENE TRANSCRIPTION AND IMPAIRS MITOCHONDRIAL FUNCTION IN THE CNS

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Abstract

Domoic acid is an algal-derived seafood toxin that functions as a glutamate agonist and exerts excitotoxicity via overstimulation of glutamate receptors (AMPA, NMDA) in the central nervous system (CNS). At high (symptomatic) doses, domoic acid is well-known to cause seizures, brain lesions and memory loss; however, a significant knowledge gap exists regarding the health impacts of repeated low-level (asymptomatic) exposure. Here, we investigated the impacts of low-level repetitive domoic acid exposure on gene transcription and mitochondrial function in the vertebrate CNS using a zebrafish model in order to: (1) identify transcriptional biomarkers of exposure; and (2) examine potential pathophysiology that may occur in the absence of overt excitotoxic symptoms. We found that transcription of genes related to neurological function and development were significantly altered, and that asymptomatic exposure impaired mitochondrial function. Interestingly, the transcriptome response was highly variable across the exposure duration (36 weeks), with little to no overlap of specific genes across the six exposure time points (2, 6, 12, 18, 24, and 36 weeks). Moreover, there were no apparent similarities at

any time point with the gene transcriptome profile exhibited by the glud1 mouse model of chronic moderate excess glutamate release. These results suggest that although the fundamental mechanisms of toxicity may be similar, gene transcriptome responses to domoic acid exposure do not extrapolate well between different exposure durations. However, the observed impairment of mitochondrial function based on respiration rates and mitochondrial protein content suggests that repetitive low-level exposure does have fundamental cellular level impacts that could contribute to chronic health consequences.

Introduction

Domoic acid is a small neurotoxic molecule that is naturally produced by phytoplankton and accumulates in seafood products during harmful algal blooms (HABs) (Wright et al., 1989). At high doses, it is responsible for the human neurotoxic illness known as amnesic shellfish poisoning (ASP), with symptoms that include seizures, memory loss, coma, and death (Quilliam and Wright, 1989). The toxin was first identified in 1987 when over 100 people became ill and four died after consuming domoic acid-contaminated mussels (Perl et al., 1990). Domoic acid has since been shown to severely impact seabirds and marine mammals via transfer through planktivorous prey (Scholin et al., 2000; Work et al., 1993). For humans, risks of exposure to high levels of domoic acid are managed by testing shellfish and regulating harvests based on toxin loads (≥20 µg domoic acid/g = harvest closure); however, there are no regulatory guidelines in place for protection from long-term low-level repetitive exposure (<20 µg domoic acid/g = harvest open) due to a lack of

knowledge regarding the health impacts of chronic or episodic low level domoic acid exposure (Lefebvre and Robertson, 2010).

The knowledge gap regarding health impacts of low-level repetitive exposure to naturally produced algal toxins, such as domoic acid, is particularly alarming in light of the increasing emergence, magnitude and frequency of domoic acid-producing HABs globally – a pattern that is predicted to worsen with continued climate change (Van Dolah, 2000). Of particular concern are coastal and tribal communities that rely heavily on seafood products as a major food source. For example, in the Pacific Northwest region of the US, the Quinault Tribe has a strong cultural reliance on razor clams for subsistence and ceremonial harvests (Lefebvre and Robertson, 2010). These clams are known to retain low levels of domoic acid for up to a year after a HAB and are eaten on a year-round basis in these communities, thereby presenting a risk of low level repetitive exposure to the consumer (Wekell et al., 1994). Further risks to the developing fetus or nursing offspring have also been identified in naturally exposed marine mammals and in laboratory studies with rodent and primate models (Gulland et al., 2002; Levin et al., 2006).

Domoic acid functions as a glutamate agonist and exerts excitotoxicity via overstimulation of glutamate receptors (AMPA, NMDA) in the central nervous system (CNS) (Berman and Murray, 1997). Domoic acid-mediated increases in glutamatergic activation may alter cellular transcriptional responses that if gene product formation follows transcriptional changes, would lead to changes in synapse structure and function, dendrite and nerve terminal integrity, and may produce neuronal injury. Changes in synaptic function and loss of select glutamatergic neurons, or their synapses, occurring during neurodevelopment or in the aged may

be particularly deleterious for normal CNS function (Morrison and Hof, 1997). Potential health risks of chronically elevated glutamatergic activity, like those that might result from chronic/episodic DA exposure, are also supported by the studies of a transgenic mouse model selectively overexpressing neuronal glutamate dehydrogenase (*Glud1*). Bao et al. (2009) and Wang et al. (2010) demonstrated that chronic moderately elevated synaptic glutamate in the *Glud1* mice leads to reductions in glutamate synapses in specific (vulnerable) brain regions, with reductions in glutamate synapses increasing with advancing age (Bao et al., 2009). Notably, these decreases in glutamate synapses were associated with upregulation of cellular pathways associated with nervous system development, neuronal growth, and synaptic transmission, as well as oxidative stress, cell injury, and inflammation, indicating apparent compensatory responses to stress that promoted growth of neuronal processes (Wang et al., 2010).

In the present study, we investigated the impacts of low-level repetitive domoic acid exposure on gene transcription and mitochondrial function in the vertebrate CNS using the zebrafish (*Danio rerio*) laboratory model in order to (1) identify transcriptional biomarkers of exposure and (2) examine potential pathophysiological impacts that may occur in the absence of overt excitotoxic behaviors. We examined temporal patterns in the transcriptome response over a 36-week domoic acid exposure period to determine whether asymptomatic exposure elicited transcriptional changes in genes related to neuronal damage and compensatory cellular responses in the CNS.

Methods

Zebrafish and chronic domoic acid exposures

Wild-type zebrafish (*Danio rerio*, AB strain), approximately5 months of age, were obtained from Oregon State University (Sinnhuber Aquatic Research Laboratory Corvallis, OR). Fish were maintained at the Northwest Fisheries Science Center (NWFSC, Seattle, WA) in a ZebTec stand-alone recirculating and continuously monitored zebrafish rack system with UV sterilizer (Techniplast, Exton, PA). Water temperature was maintained at 26°C, and fish were kept on a 12:12 h light:dark cycle and fed daily with BioVitafish feed (Bio-Oregon, Longview, WA). Starting at ~7 months of age, zebrafish were repeatedly exposed to asymptomatic doses of domoic acid via intracoelomic injection (10 µL volume delivered with 33-gauge needle in custom-made auto-injector from Hamilton®) for 36 weeks. Toxin and vehicle (PBS) injections were given once a week (dose = 0.31 ± 0.03 µg domoic acid/g fish) for the first 6 weeks, then once every 2 weeks (dose = 0.18 ± 0.02 µg domoic acid/g fish) for the duration of the exposure period. After each injection, fish were observed for 30–45 min to note the instance of any neurobehavioral symptoms (e.g., circle- or spiral-swimming). Domoic acid doses of less than half of the previously reported EC_{50} for zebrafish (0.86 µg domoic acid/g fish; Lefebvre et al., 2009) were chosen to ensure asymptomatic exposures. All domoic acid dose concentrations were verified via HPLC as described in Lefebvre et al. (2009). Fish (n = 9–10 per treatment group per time period) were harvested at six time points, including 2, 6, 12, 18, 24 and 36 weeks exposure for whole brain transcriptome analyses. Additionally, whole fish (n ≈ 10 per treatment) at the 36-week exposure time point were preserved for histopathological analysis.

Histopathological examination

Zebrafish allocated for histopathological examination (n ≈ 10per treatment) at the 36-week exposure time point were examined grossly for any externally visible abnormalities, placed in an ice bath and exsanguinated via a tail bleed to obtain serum for another study. Fish were then carefully opened with a midline incision of the abdomen, and the entire fish was preserved in 20 mL Dietrich's fixative (Fournie et al., 2000) at an ~1:20 (v/v) tissue to fixative ratio and fixed for three days on an orbital shaker. After fixation, the fish were rinsed in 2–3 changes of water and placed in 70% ethanol. Fish were bisected along the sagittal plane using a razor blade, cutting parallel to and on the left side of the spinal cord, before they were processed. The bisected fish were loaded into cassettes for processing, using a Shandon Hypercenter XP tissue processing center. The tissue processing protocol closely followed that specified by the Zebrafish International Research Center, University of Oregon, Eugene, OR (http://zfin.org/zirc/disMan/diseaseManual.php). Processed tissues were infiltrated with a 50:50 ratio of Fisher Paraplast PLUS tissue embedding medium and Surgipath Formula 'R' infiltrating and embedding paraffin. Tissues were then embedded in Surgipath Formula 'R' infiltrating and embedding paraffin, using a Shandon Embedding Center.

Fixed, paraffin-embedded fish were cut in the sagittal plane with a high-profile disposable blade in multiple, stepped-sections (~20 µm apart) at 5–7 µm thickness. Multiple sections of gills, eyes, brain, spinal cord and all internal organs were taken to ensure complete histopathological evaluation. Sections were routinely stained by hematoxylin and eosin, using the protocols described in "Diseases of Zebrafish in Research Facilities" (Zebrafish International Research Center). Sections were

examined by light microscopy on a Nikon Optiphot microscope, using 4×, 10×, 20×, 40×, and 100× planachromat objectives; selected sections of the central nervous system were photographed by digital photomicroscopy on a Nikon Eclipse E600 microscope, using a SPOT^{TM} camera and SPOT^{TM} soft-ware. All major organs were examined for pathological changes, with a focus on alterations in components of the central nervous system. Any observed anomalies were scored by type, distribution within the tissue (e.g., focal/multifocal/diffuse) and severity, using a semiquantitative, ordinal scoring system detailed in Fairgrieve et al. (2005). Potential lesions/anomalies of interest were shared as needed with other fish histopathologists experienced in the examination of zebrafish (Dr. Trace Peterson and Dr. Michael Kent of Oregon State University) for confirmation and verification.

Brain dissections and RNA extraction

Zebrafish were euthanized via decapitation and whole brains were dissected from the fish as described in Lefebvre et al. (2009).Global transcriptome expression was quantified by microarray in three RNA replicates, each consisting of RNA from 3 pooled brains, for exposed and control treatments. The test group size (n = 9–10 per treatment per time point) was chosen based on a previous study (Lefebvre et al., 2009). Total RNA was isolated from zebrafish brains using the miRNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to the vendor's defined method, and stored at −70°C. RNA quantity (ng/µL) was determined by measuring the OD_{260} with a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA); RNA purity was assessed by measuring $OD_{260/280}$ and $OD_{260/230}$ ratios. RNA integrity (quality) was characterized using the Agilent RNA 6000 Nano Kit with an Agilent 2100 Bio-analyzer (Agilent Technologies, Santa Clara, CA). Only total RNA samples

with appropriate size distribution, quantity, and $OD_{260/280}$ as well as $OD_{260/230}$ ratios of 1.8–2.1 were used for microarray-based analysis. Individual brain RNA samples were split, and half was used for microarray analyses and half retained for RT-PCR confirmation.

Global transcriptome profiling in brain

The RNA samples were labeled and prepared for hybridization onto a Zebrafish (V3) Gene Expression Microarray (Agilent Technologies, Inc. Santa Clara, CA) using the manufacturer's established protocols. Hybridization and washing of these arrays was accomplished using HS 400 Pro hybridization and wash stations (Tecan Systems, Inc., San Jose, CA) and scanned using an Agilent DNA Microarray Scanner (Agilent Technologies, Inc. Santa Clara, CA), according to the manufacturer's established standard protocol. In total, 36 microarrays were used, including 18 for the control group and 18 for the chronic exposure group. Separate pools of RNA, each consisting of three individuals, were hybridized to each array resulting in three biological replicates (a total of nine individuals) per treatment group at each time point.

Microarray analyses

Raw microarray data were generated with the Agilent Feature Extraction image analysis software (Agilent), and further processed and analyzed with tools from Bioconductor (Gentleman et al., 2004). Data were normalized using the BioconductorAgi4644Pre-Process package (Smyth, 2004); additionally, the normexp option for background adjustment and quantile normalization was used for the between array normalization step. Using the normalized data, genes with significant

evidence for differential expression were identified using the limma package. The limma methodology calculates a P-value for each gene using a modified t-test in conjunction with an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. This method of detecting differentially expressed genes draws strength across genes for more robust and accurate detection of differentially expressed genes; such an adjustment has been shown to avoid an excess of false positives when identifying differentially expressed genes (Allison et al., 2006). Using the P-values from limma, the Bioconductor package p.adjust (Benjamini and Hochberg, 1995) was used to estimate the false discovery rate associated with the list of differentially expressed genes. This methodology allows us to address the multiple testing problems without resorting to an excessively conservative approach that controls the family-wise error, such as a Bonferroni correction. Comparative analyses on bio-functions of the differentially transcribed genes were conducted using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA; Build Version 242990, Content Version 12710793 (Release date 2012-05-07)). IPA uses a Fischer's exact test to calculate the P-values. All microarray data are available through the NCBI Gene Expression Omnibus database (accession #: GSE34716).

Quantitative RT-PCR analyses

Six neurodevelopment-related genes belonging to biological functions categories: nervous system development and function; neurological disease; cell death; cellular function and maintenance; and cellular growth and proliferation were further validated by RT-PCR in samples from all time points (6, 12, 18, 24 and 36weeks). Total RNA was isolated from individual zebrafish brains (n = 9 per

treatment per time point). RT-PCR was performed on pooled samples as described for microarray analyses (i.e., n = 3 brains per pooled sample, yielding n = 3 independent samples per treatment per time point). Briefly, reverse transcription was performed according to the manufacturer's established protocol using total RNA and the SuperScriptH III First-Strand Synthesis System (Invitrogen, Carlsbad, CA.). For gene expression measurements, 2 µL of cDNA were included in a PCR reaction (12 µL final volume) that consisted of the ABI inventoried TaqManH Gene Expression Assays mix (Applied Biosystems Inc., Foster City, CA), or customized forward and reverse primers, probes and TaqMan Gene Expression Master Mix (Applied Biosystems Inc., Foster City, CA). The PCR primers and the dual-labeled probes for the genes were designed using ABI Primer Express v.1.5 software (Applied Biosystems Inc., Foster City, CA). Amplification and detection of PCR amplicons were performed with the ABI PRISM 7900 system (Applied BiosystemsInc., Foster City, CA) with the following PCR reaction profile: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 30 s, and 60°C for 1 min. Beta-actin 1 amplification plots derived from serial dilutions of an established reference sample were used to create a linear regression formula in order to calculate expression levels, and Beta-actin1 gene expression levels were utilized as an internal control to normalize the data.

Western blotting of oxidative damage and mitochondrial proteins

In situ mitochondrial respiration was measured in whole brains of control and exposed zebrafish at 18 weeks of chronic asymptomatic exposure. Fish were euthanized via decapitation and whole brains were dissected and either placed in ice-cold respiration buffer for mitochondrial respiration (n = 6 per group) or homogenized for western blotting (n = 11 per group) of protein carbonyls and

electron transport chain proteins as described previously (Siegel et al., 2012). Briefly, homogenates were diluted 1:25 in lysis buffer containing protease inhibitors, combined 1:1 with Laemli buffer and separated on a 4–20% gradient gel. Proteins were transferred to nitrocellulose membrane and Ponceau stained to visualize protein loading and immunoblotted as follows: complex II 30 kDa subunit (1 h 1:1 K Mitosciences #203 in 5% NFDM), complex IV subunit IV (1 h 1:1 K Mitosciences #407 in 5% NFDM), and protein carbonyls (according to Millipore kit S7150). Secondary Ab from Cell Signaling (#7076, 1:10 K in 1% NFDM) was conjugated to horseradish peroxidase. Blots were developed in Western C chemiluminescent developer (BioRad, Hercules, CA) and visualized with a ChemiDoc imaging system. Band densities were quantified with Quantity One software and normalized to total lane Ponceau staining.

Quantification of mitochondrial respiration

Mitochondrial respiration was measured in whole brain homogenates in respiration buffer (210 mM sucrose, 0.5 mM EGTA, 3 mM $MgCl_2$, 10 mM KH_2PO_4, 20 mM HEPES, 20 mM taurine, 50 mMK-MES, 1 g/L BSA, pH = 7.1) using a clark-type electrode in an Oxygraph 2 K (Oroboros Instruments, Austria). Multiple respiration states were measured using the following substrate inhibitorcombinations: proton leak (state 4; 10 mM glutamate/5 mM pyruvate/2 mM malate, without ADP), state 3 (state 4 conditions plus1 mM ADP with and without 10 mM succinate), fully uncoupled (ETS, 1 µM CCCP) or complex IV (0.5 mM N,N,N',N'-tetramethyl-ρ-phenylenediamine (TMPD), 2 mM ascorbate, 2.5 µm antimycin A). Potassium cyanide (2 µM) was used to confirm complex IV-specific activity. The amount of oxygen consumed was calculated by assuming the O_2 solubility in media to be 0.920

and by calibrating initial oxygen concentration in the buffer for each experiment and correcting for pressure, temperature and instrumental oxygen consumption as described by Gnaiger (2009).

Results

Chronic low-level domoic acid exposure did not cause neurobehavioral excitotoxicity or visible histopathological lesions

The domoic acid exposure regimen used here did not produce any visible signs of neurobehavioral excitotoxicity (i.e., circle- or spiral-swimming) when observed over a 30–45 min period following each injection, consistent with an asymptomatic exposure regimen (data not shown). Because domoic acid is well-known to produce excitotoxic neuron death and histopathological lesions in glutamate-rich brain areas, we examined whether there was evidence of lesions in sagitally sectioned brains of control and exposed zebrafish from the 36-week exposure time point. Semi-quantitative scoring of the brain and spinal cord revealed no visible histological differences between control and exposed fish (Fig. 1). All other major organ systems were also examined and appeared normal (data not shown). Consistent with our histopathological analysis, microarray analysis of *gfap* gene expression, a marker of neuroinflammation and astrocyte activation, revealed no significant changes (Table 1).

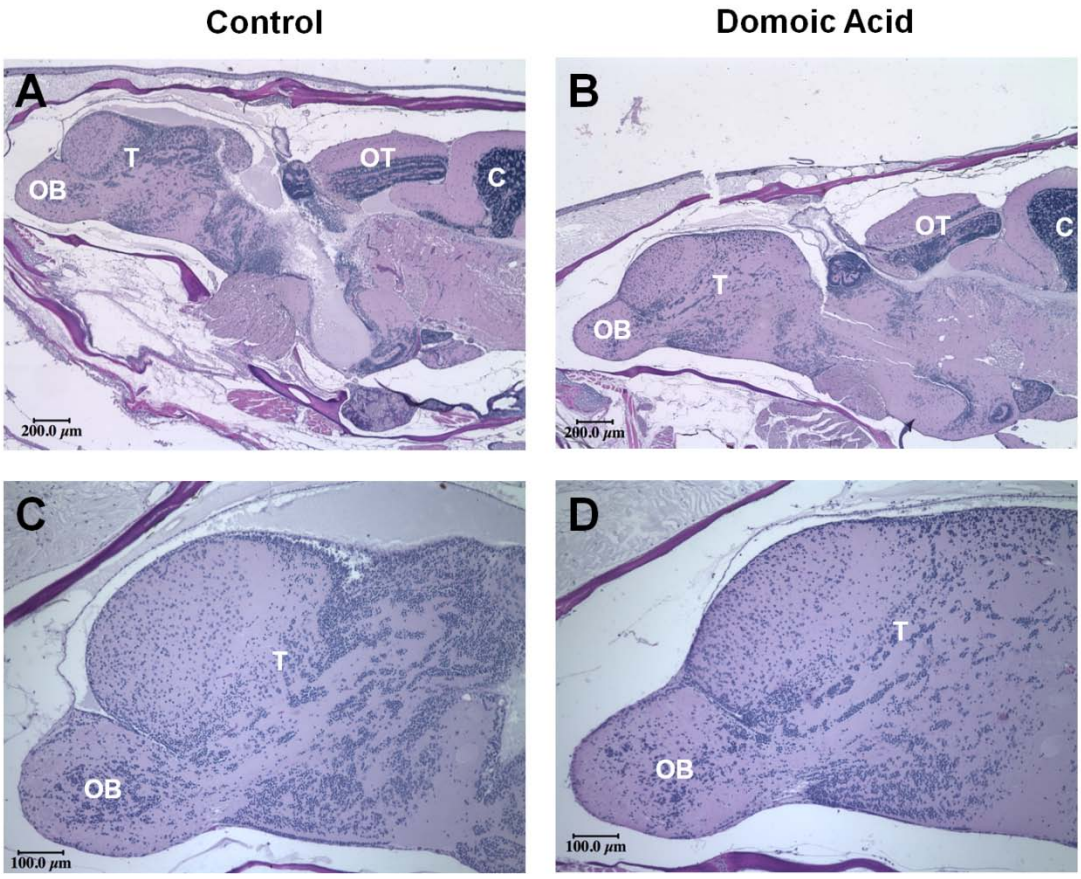

Figure 1. Representative H&E-stained sagittal sections of control (A and C) and domoic acid-exposed (B and D) zebrafish at 36 weeks. Whole brain images (A and B) include olfactory bulb (OB), telencephalon (T), optic tectum (OT) and anterior cerebellum (C) (4×); panels (C) and (D) depict olfactory bulb (OB) and telencephalon (T) (10×). The telencephalon phylogenetically develops into the brain region that includes the hippocampus in higher-order organisms.

Table 1. Gene transcription data for *gfap* (glial fibrillary acidic protein; $\log_2$(fold-change), *p* value), a marker of astrocyte activation and neuroinflammation, as measured by microarray. Transcriptional changes are shown for animals exposed to domoic acid relative to the time-matched vehicle controls. Note that all array probes for this gene are non-significant, highlighting the absence of gross neuroinflammation and corroborating the histopathological assessment presented in Fig. 1.

		***gfap* Log$_2$(fold-change) (*p value*)**					
NCBI GenBank ID	Probe ID	2 weeks	6 weeks	12 weeks	18 weeks	24 weeks	36 weeks
NM_131373	A_15_P107505	0.33 (*0.19*)	0.02 (*0.93*)	0.14 (*0.56*)	0.45 (*0.07*)	0.13 (*0.59*)	-0.14 (*0.57*)
NM_131373	A_15_P131871	0.02 (*0.77*)	-0.03 (*0.65*)	0.03 (*0.64*)	0.05 (*0.47*)	-0.03 (*0.67*)	-0.07 (*0.30*)
AY397679	A_15_P551122	0.00 (*0.90*)	0.01 (*0.78*)	-0.01 (*0.71*)	-0.02 (*0.30*)	0.00 (*0.88*)	-0.01 (*0.62*)
NM_131373	A_15_P658936	0.05 (*0.57*)	-0.01 (*0.91*)	0.04 (*0.59*)	0.19 (*0.02*)	0.03 (*0.73*)	-0.05 (*0.55*)

Chronic low-level domoic acid exposure led to temporally variable gene transcription responses

The number of significantly differentially transcribed genes (±1.5-fold-change compared to control, $p < 0.05$) ranged from 52 to 239 at each time point, with the majority of genes having increased transcription (60–64%) (Fig. 2A). Notably, the vast majority (77–94%) of differentially transcribed genes were unique to a particular time point, and very few individual genes were differentially transcribed across multiple time points (Fig. 2A). In order to characterize broad temporal patterns in the transcriptome response to chronic domoic acid exposure, we performed gene set analysis (GSA) on grouped time points (early = 2 and 6 weeks; mid = 12 and 18 weeks; late = 24 and 36 weeks); time points were grouped in order to achieve improved statistical power for GSA. The heatmap in Fig. 2B illustrates these grouped time points, underscoring the temporally dynamic gene transcription profile over the

duration of domoic acid exposure, suggesting that the gene transcriptome response to chronic domoic acid exposure depends largely on the exposure duration.

While GSA revealed a broad range of significantly altered Biological Processes & Molecular Functions (as defined by gene ontology), no clear temporal patterns emerged from this analysis (data not shown). That is, we were unable to detect any transcriptional effect aligned with our expectation that early response (2 and 6 weeks exposure duration) would transition into a consistent response profile later in the chronic exposure paradigm (12, 18, 24 and 36 weeks).

In order to gain a more function-based understanding of pathways and biological functions affected by chronic domoic acid exposure, we also performed Ingenuity Pathway Analysis (IPA) of gene expression at each individual exposure duration time point. Our primary hypothesis entering the study was that chronic asymptomatic domoic acid exposure would alter the transcription of genes associated with cellular death/damage and neurological function/development. Ingenuity Pathway Analysis identified 9–70 significant biological function categories at each time point, with categories defined here as containing ≥3 genes (data not shown). The Bio Functions most relevant to our hypothesis, i.e., nervous system development & function, neurological disease, cell death, cellular growth and proliferation, and cellular function and maintenance, are shown in Fig. 3 (full gene list in Table 2). Notably, these IPA Bio Functions were significantly altered by domoic acid exposure at five to six of the six exposure duration time points (Fig. 3). However, while there is overlap of individual genes across Bio Functions within each time point (~73–97% overlap between Bio Function genes within individual time points), there is very little overlap of individual genes across individual time points

(e.g., only ~2 genes within a Bio Function category appear at two or more time points). This further supports the finding that the transcriptome response to chronic domoic acid is highly temporally variable and dynamic.

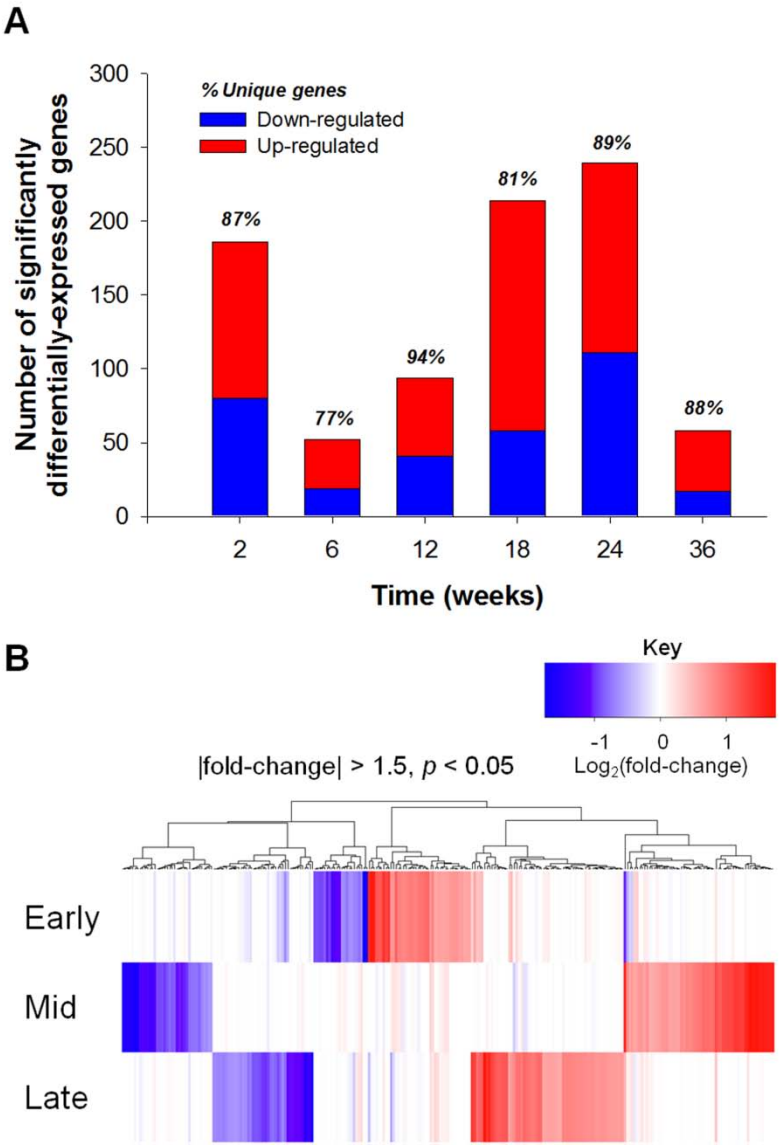

Figure 2. (A) Number of genes significantly differentially regulated throughout duration of chronic exposure to asymptomatic domoic acid compared to time-matched vehicle controls. The proportion of up- and down-regulated genes is shown by red and blue bars, respectively. Percentage of genes unique to each time point is listed above each bar. (B) Heatmap depicting clustered gene transcription responses (±1.5-fold, p < 0.05, 265 probes) to chronic domoic acid exposure across exposure duration; early = 2 and 6 week time points, mid = 12 and 18 week time points, late = 24 and 36 week time points.

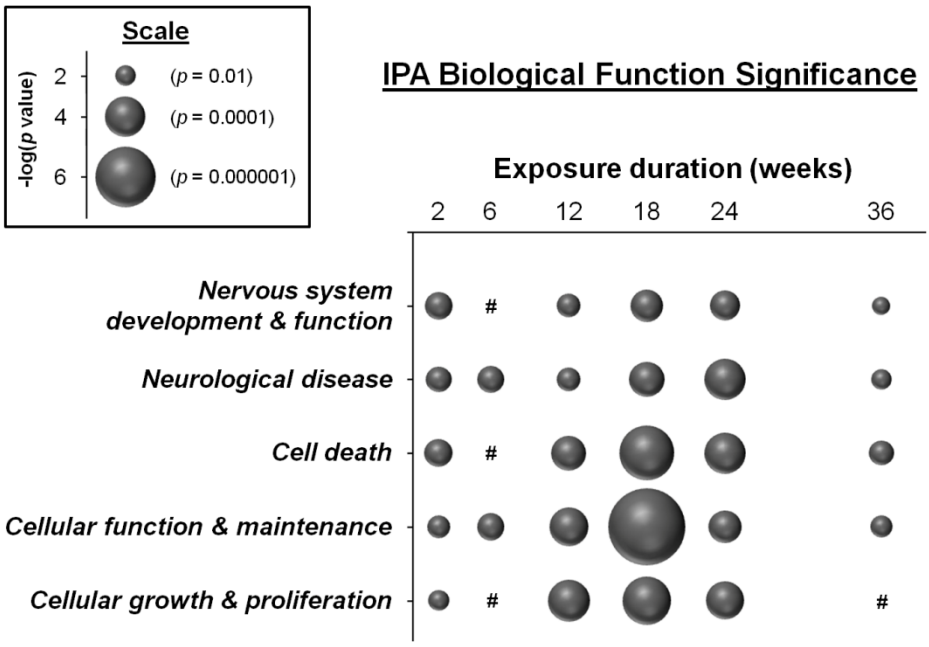

Figure 3. Significantly altered ($p < 0.05$) biological functions (from Ingenuity Pathway Analysis) associated with cellular and neurological health, function and development. Bubble diameter corresponds to −log(p value) for functional categories and time points possessing ≥3 genes; # indicates not significant and/or <3 genes within the category. Scale provides reference for relative bubble diameter.

Table 2. All genes falling into the bio function categories shown in Fig. 3 at all time points. (*Continued on next page.*)

Category	Genes	
Cell Death (92)	**Up-regulated:**	*ADIPOQ, AGGF1, APOE, APP, ARG1, ATG3 (includes EG:171415), B2M, B4GALNT1, BCL2L1, BNIP3, RFWD2, CCNI, CCT7, CD53, CD74, CDC73 (includes EG:214498), CHFR, CORO1A, COX5A , includes EG:12858), CTSB, CTSD, CTSS, CYLD, DRAM1, DUSP2, ELMO1, EPO, FKBP5, GAS6, GMFG, GRN, HAND2, HBZ, HDAC5, AGT, HLA-DRB1, IRF1 (includes EG:16362), IRF8, JAG1, LCP1, LECT2, LGALS3, LGALS3BP, MCL1, MCM10 (includes EG:307126), MEF2C, MT2A, NFKBIA, NTRK3, NUDCD3, PPP1CA, PRKCD, PSME2, RBBP4, SOCS1, SOCS3, TAP2, TFDP1, TOP1, TRIM2, UCP2, USP2, VDAC2, WAS, WEE1*
	Down-regulated:	*AKT1, ANXA11, ATP2A1, AURKA, CALCB, CAV1, CCDC47, CGA, CHKA, CLCN3, DYRK1B, FAH, GH1, GRIA2, LCK, LIFR, NOD1, P4HB, POMC, PPID, PPM1L, PTGES, RFXANK, SH3BP5, SIRT2, TASP1, TSG101*

Cellular Function and Maintenance (81)	**Up-regulated:**	*ADIPOQ, AGT, APOE, APP, ATP6V1B2, B2M, B4GALNT1, BCL2L1, BNIP3, CACNB3, CD74, CORO1A, COX5A (includes EG:12858), CTSD, CTSS, CYLD, DGKA, DRAM1, DUSP2, ELMO1, EPO, GAS6, GPR12, GRN, HDAC5, HIVEP2, IRF1 (includes EG:16362), IRF8, JAG1, LCP1, LECT2, LGALS3, MCL1, Mcpt4, MGAT5, MT2A, NEO1, NFKBIA, NIN, NTRK3, PPP1CA, PRKCD, PSMB10, PSME2, RBBP4, RHCG, SLC9A3 (includes EG:105243), SOCS1, SOCS3, STK35, Tlr12, TOP1, TPM2, UCP2, UCP3, WAS*
	Down-regulated:	*AKT1, ATP2A1, BCL11A, CALCB, CAV1, CGA, CLCN3, CREB3L2, CUBN, CYP4A11, GH1, GNB1, GORASP1, LCK, LIFR, NEFM, NOD1, PAK7, POMC, PPID, PPP1CA, SASH3, SLC25A47, SPC25 (includes EG:100144563), TSG101, ULK1*
Cellular Growth and Proliferation (49)	**Up-regulated:**	*ADIPOQ, AGT, APOE, APP, B2M, B4GALNT1, CD74, CDC73 (includes EG:214498), CORO1A, CTSB, CTSD, DUSP2, EPO, GAS6, GRN, HLA-DRB1, IRF1 (includes EG:16362), IRF8, JAG1, MCL1, MKNK1, MT2A, NFKBIA, NR1D1, PRKCD, PSMB10, PSMB9, RAD17 (includes EG:19356), SOCS1, SOCS3, TPM2, WAS*
	Down-regulated:	*AKT1, CAV1, CD151, CGA, CHKA, DYRK1A, DYRK1B, GH1, IK, LCK, LIFR, PKP3, POMC, PTGES, RAB5C, SIRT2, TSG101*
Nervous System Development and Function (41)	**Up-regulated:**	*AGT, APOE, APP, B4GALNT1, BCL2L1, CACNB3, CTSF, EPO, FEV, FGFRL1, GAS6, GJA8, GNGT1, MT2A, NFKBIA, NR1D1, NRXN2, NTRK3, PAK4, PDE6G, RPE65, TRIM2*
	Down-regulated:	*AKT1, ALS2CR8, BCL11A, CAV1, CGA, CLCN3, DYRK1A, FZR1, GH1, GRIA2, LIFR, NEFM, PAK7, POMC, PPP1CA, RBP4, RTN4RL1, SIRT2, ULK1*
Neurological Disease (62)	**Up-regulated:**	*ADIPOQ, ALDH7A1, APOE, APP, ARG1, ATP1B1, ATP6V1B2, B2M, B4GALNT1, BCL2L1, CD74, CORO1A, CTSB, CTSD, CTSF, CTSS, ELMO1, EPO, GNGT1, GRN, HLA-DRB1, HNRNPU, IDH2, IRF1 (includes EG:16362), MGAT5, MKNK1, MT2A, NFKBIA, PCSK5, PDE6H, PSMB9, PSME1, RARS2, RPE65, SLC1A4, SOCS3, SRD5A1, TAP2, TPM2*
	Down-regulated:	*ACOX1, AKT1, ANKRD11, ATP2A1, BEST1, CAV1, CDC73 (includes EG:214498), CGA, CLCN3, CLEC3B, GH1, GRIA2, IMPDH1, MYH7, NEFM, PER2, PPID, PTGES, RNASET2, SEPP1, SRM, TASP1, TEAD1*

Real-time quantitative PCR confirms microarray gene transcription results

Six genes (*gria2a*, *nrxn2a*, *appa*, *nfkbiab*, *bcl2L1*, and *pak7*) were used for RT-PCR confirmation at all time points (2, 6, 12, 18, 24, 36 weeks). Eighty percent of the genes across all 36 comparisons agreed in fold-change direction.

Chronic low-level domoic acid exposure led to oxidative damage and mitochondrial dysfunction

To test whether chronic domoic acid exposure resulted in changes in cell function, we measured oxidative damage to proteins, mitochondrial protein content, and mitochondrial respiration rates in the brains of zebrafish following 18 weeks of exposure to asymptomatic doses of domoic acid. Protein carbonyls (Fig. 4A), a measure of oxidative damage, were significantly elevated in whole brains. Electron transport chain (ETC) complex II and complex IV subunit expression were significantly higher in the domoic acid exposed zebrafish brains (Fig. 4B). Despite the increase in mitochondrial proteins, there were no significant differences in mitochondrial respiration per unit tissue (Fig. 4C). However, maximum mitochondrial respiration rates normalized to complex IV and complex II protein expression (Fig. 4D) were significantly decreased in the brains of domoic acid-exposed fish compared to saline-exposed controls. These results suggest that chronic low-level exposure to domoic acid leads to mitochondrial dysfunction that is partially offset by increases in mitochondrial content.

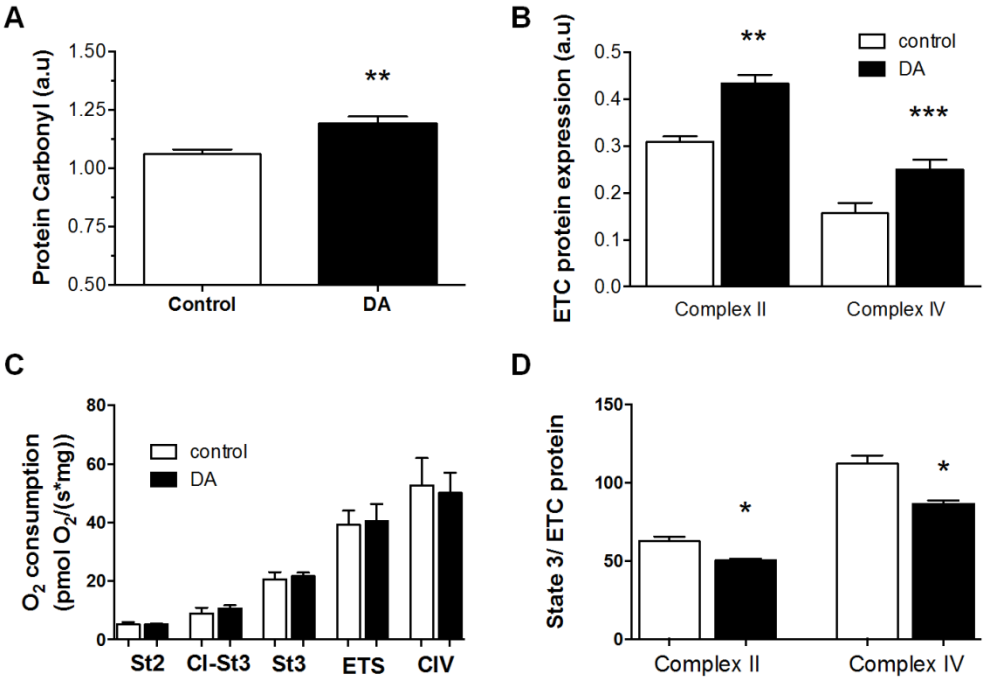

Figure 4. Comparison of oxidative damage and mitochondrial content and function in brains of zebrafish exposed for 18 weeks to domoic acid and time-matched vehicle controls. (A) Protein carbonyl levels were significantly higher in exposed fish compared to controls. (B) Expression of complex II (30 kD subunit) and complex IV (subunit IV) were both significantly higher in exposed fish compared to controls. (C) Oxygen flux per unit brain tissue at five different measures of mitochondrial respiration (St4 = state 4, C1-St3 = Complex I State 3, St3 = Complex I & II State 3, ETS = fully uncoupled respiration, and CIV = Complex IV) was not significantly different between control and exposed fish. (D) Maximal state 3 respiration (ADP, plus succinate and glutamate/malate) was normalized to complex II and complex IV protein content. Flux per content was reduced in exposed fish compared to controls. n = 6–11 for control and exposed treatments, mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$ relative to controls.

<u>Chronic low-level domoic acid exposure led to unique transcriptome responses compared to an elevated glutamate release exposure model</u>

To test our hypothesis that chronic asymptomatic domoic acid exposure in zebrafish produces brain transcriptome-level changes consistent with chronic excess

synaptic glutamate release, we compared the gene transcriptome changes in zebrafish with reported gene transcriptome changes in the hippocampi of *Glud1*transgenic mice (Wang et al., 2010). This mouse model was developed in order to study the neurological consequences of excess glutamatergic stimulation, and constitutively over-expresses a copy of the *glud1* gene, leading to moderate over-production of glutamate and increased (~30%) release of glutamate during synaptic transmission (Bao et al., 2009). For our analysis, we compared the significance and directionality of zebrafish/mouse homologues, as well as Biological Functions identified by IPA. We identified 459 zebrafish homologues of the 1018 *Glud1* genes that were differentially expressed compared to wild-type. Interestingly, only six of those zebrafish homologues were significantly differentially regulated with chronic domoic acid exposure in zebrafish at any of the exposure duration time points; of those six genes, four were altered in the same direction as their *Glud1* mouse counterparts (Fig. 5). Two Biological Functions, as identified by IPA, were found in common between the *Glud1* mouse model analysis and our targeted analysis of the zebrafish brain transcriptome: Nervous System Function & Development; Cell Death. Genes differentially transcribed at multiple domoic acid-exposure duration time points in adult zebrafish whole brain do not correspond to genes differentially transcribed in the nine month-old *glud1* mouse hippocampus.

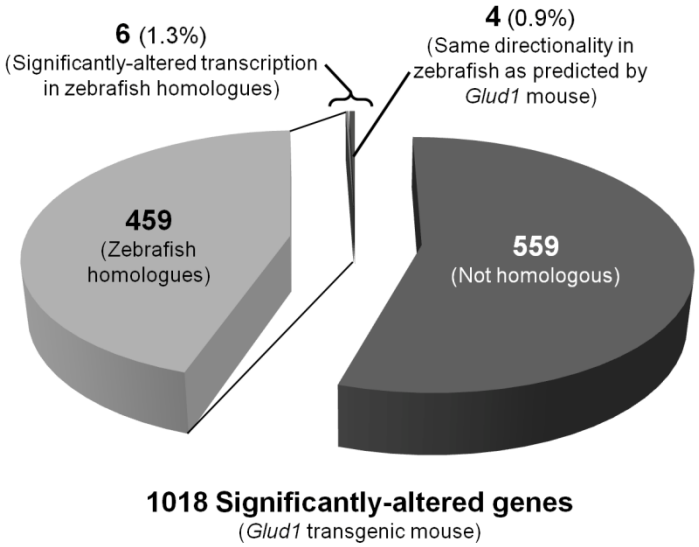

Figure 5. Comparison of transcriptome responses in zebrafish whole brain and *Glud1* transgenic mouse hippocampus. Number of genes for each wedge is indicated. Percent of zebrafish homologues is listed parenthetically. Of the 459 zebrafish homologues identified, only 6 were significantly altered; of those 6 genes, 4 were regulated in the same direction as in the *Glud1* hippocampus.

Discussion

In order to elucidate the neurological consequences of chronic exposure to low-level (asymptomatic) domoic acid doses, we conducted a transcriptome profiling analysis on the brains of zebrafish exposed for 2–36 weeks. We hypothesized that: (1) chronic asymptomatic domoic acid exposure would lead to a consistent gene transcription profile in the brains of exposed zebrafish; and (2) this profile would be similar to the gene transcription profile in the hippocampi of *Glud1* transgenic mice, a model of chronic moderate glutamatergic over-stimulation (Bao et al., 2009; Wang et al., 2010). We found that genes and biological functions related to neurological function and development were significantly altered; however, the transcriptome response was highly variable across exposure duration, with little to no overlap of

specific genes across the six exposure duration time points. Additionally, while biological function categories were similar between exposed zebrafish and the Glud1 mouse model, less than 1% of individual genes overlapped between models.

Chronic low-level domoic acid exposure does not lead to a consistent transcriptome profile

We expected to identify early changes in gene transcription following 2–6 weeks of domoic acid exposure, leading to a consistent response at the later chronic time points. Instead, we found that the response was extremely variable and dynamic across all exposure duration time points. The number of differentially transcribed genes unique to each time point ranged from 77 to 94% (Fig. 2A), with only three genes significantly regulated at more than two time points. This shows that prolonged asymptomatic domoic acid exposure produced a continuum of diverse gene transcription responses.

This study is highly unique in its ability to evaluate the temporal nature of the transcriptome response because it assessed six time points across a duration that spanned a time period equivalent to one-third of the organism's life. Prior studies have typically investigated gene transcription changes following only an acute exposure to symptomatic doses of domoic acid (Ananth et al., 2001; Ryan et al., 2005). Additionally, our previous work has shown that whole-brain transcriptional response to symptomatic doses of domoic acid differs markedly from the transcriptional response to asymptomatic exposure (Lefebvre et al., 2009). Our current results suggest that studies utilizing only a single domoic acid exposure duration may observe transcription profiles unique only to that particular time point, and may not extrapolate to other exposure durations.

Chronic low-level domoic acid exposure alters transcription of genes related to nervous system function and development

We hypothesized that chronic asymptomatic domoic acid expo-sure would lead to transcriptional changes in genes related to neurological homeostatic function and development based on (1) evidence that perturbation to the glutamate system is linked to several neurological disorders (Choi, 1988); and (2) a model of differential neural insult whereby subtle insults, similar to chronic asymptomatic domoic acid exposure, lead to 'alerted' microglia, which contribute to a neuro-supportive environment by release of neurotrophic factors (Hanisch and Kettenmann, 2007). This is in contrast to the cascade of more overt damage caused by full activation of microglia, which can ultimately lead to astrocyte activation and neuroinflammation. Notably, *gfap* (glial fibrillary acidic protein), a marker of astrocyte activation and neuroinflammation, was not significantly up-regulated at any of the time points (Table 1), highlighting the absence of overt neuroinflammation consistent with the unremarkable histopathology reported in Fig. 1.

Ingenuity Pathway Analysis (IPA) of our datasets revealed several significantly altered transcriptional responses in genes categorized in Biological Functions relevant to our hypotheses (Fig. 3). Within the IPA Bio Function categories nervous system development & function and cell death, we examined six genes of interest, including *gria2a*, *nrxn2a*, *pak7*, *appa*, *nfkbiab*, and *bcl2L1*. At the 2-weektime point, the AMPA glutamate receptor encoded by *gria2* was down-regulated (Table 2). Notably, this was the only glutamate-related gene significantly altered by domoic acid exposure at anytime point. Down-regulation of this receptor has been shown to occur following seizure activity (Huang et al., 2002), suggesting that the

down-regulation of *gria2a* at 2 weeks is a short-term compensatory response to elevated glutamatergic activity.

Neurexin 2a gene (*nrxn2a*), which encodes a synaptic organization cell adhesion molecule, was up-regulated at 2 weeks (Table 2). Neurexins have been linked to Alzheimer's disease (Gauthier et al., 2011), and may play a role in maintaining mature neural networks (Rozic-Kotliroff and Zisapel, 2007); up-regulation at this time point suggests an alteration of synaptic signaling and/or neural disruption. A member of the PAK family of Ser/Thr protein kinases encoded by *pak7*, was down-regulated at the 36-week time point (Table 2). *Pak7* is involved in neurite outgrowth (Dan et al., 2002) and its disruption suggests that synaptic trafficking may be altered (Strochlic et al., 2012) following prolonged exposure to domoic acid.

Transcriptional responses of a trio of genes involved in neuroprotective, anti-inflammatory, and apoptotic responses, *appa*, *nfkbiab*, and *bcl2L1*, were significantly up-regulated following 24weeks of domoic acid exposure (Table 2). Amyloid precursor protein (APP) is a membrane protein that is metabolized to β-amyloid– the main component of Alzheimer's disease (AD) plaques (Klunk and Abraham, 1988). While the function of APP in the non-AD brain is unknown, it has been suggested to play a neuroprotective role under normal physiological conditions (Wentzell et al., 2012). *Nfkbia*, up-regulated 2.8-fold, encodes a cellular protein that inhibits the NF-κB transcription factor (Arenzana-Seisdedos et al., 1995), which contributes to a pro-inflammatory cascade when activated. A member of the Bcl-2 family, *bcl2L1*, was also up-regulated; the proteins in this family play a role in the regulation of apoptosis and are localized to the mitochondrial membrane (Plas and Thompson, 2002).

Chronic low-level domoic acid exposure causes oxidative damage, impairs mitochondrial function and may trigger compensatory mitochondrial biogenesis

As a glutamate agonist, domoic acid overexcites glutamatergic receptors and ultimately causes excess Ca^{2+}influx at acute doses, making mitochondria an obvious target for toxic insult due to their central role in Ca^{2+} homeostasis (Nicholls, 2009; Nijjar and Nijjar, 2000). In the present study, differential transcription of the *bcl2L1* gene, involved in mitochondrial regulation of apoptosis described above, provides evidence of mitochondrial involvement in chronic asymptomatic domoic acid toxicity. Previous studies in mice have further demonstrated that oxidative stress-induced mitochondrial dysfunction is the underlying mechanism of domoic acid-mediated cognitive deficits after acute exposure (Lu et al., 2012). Elevated protein carbonyl levels in chronically exposed zebrafish found in the present study suggest that oxidative stress also plays a role in toxicity. Additionally, enzymatic assays of the mitochondrial electron transport chain (complexes I–V) in isolated rat mitochondria showed marked concentration-dependent impairment in cardiac energetics following exposure to domoic acid (Vranyac-Tramoundanas et al., 2008); this is consistent with our findings of reduced mitochondrial-specific respiration in the brains of chronically exposed zebrafish (Fig. 4D). Our findings of increased oxidative damage (Fig. 4A) coupled with reduced mitochondrial respiration (Fig. 4D) suggest that low-level repetitive domoic acid exposure also causes oxidative stress-related mitochondrial dysfunction. Interestingly, mitochondrial content was significantly higher in exposed zebrafish (Fig. 4B), suggesting that a compensatory mitochondrial biogenesis response may be initiated to meet energy demands in a living organism. Evidence for improvement in domoic acid-induced cognitive deficits by stimulation of

estrogen receptor-α-mediated mitochondrial biogenesis signaling has been reported previously (Lu et al., 2012).

Chronic low-level domoic acid exposure leads to unique transcriptome profiles compared to the Glud1 transgenic mouse model

We anticipated that the whole-brain transcriptome profile in zebrafish chronically exposed to asymptomatic domoic acid doses would be similar to the hippocampal transcriptome response in the *Glud1* transgenic mouse model (Wang et al., 2010). This transgenic line transcribes extra copies of *Glud1* (glutamate dehydrogenase1) under the control of the promoter for neuron-specific enolase (*Nse*) in neurons. This contributes to increased glutamate release in striatal and hippocampal neurons compared to wild-type mice (Bao et al., 2009).

We compared transcriptome results from the brains of chronically exposed zebrafish to hippocampi from nine-month old *Glud1*mice (Wang et al., 2010), and identified transcriptional changes for genes from two IPA Bio Functions groups in both the present study and the *Glud1* mouse study: (1) Nervous System Function & Development; and (2) Cell Death. However, individual gene transcription responses in zebrafish were different from those reported in the *Glud1* mouse hippocampus (Fig. 5). Out of 459 zebrafish homologues to the 1018 differentially transcribed *Glud1* mouse genes, only six genes (1.3% of zebrafish homologues) were significantly differentially transcribed following domoic acid exposure, and only four (0.9% of homologues) were altered in the same direction as the *Glud1* genes. This indicates that episodic domoic acid exposure leads to a toxicological response similar to one produced by chronically moderately elevated glutamatergic activity at a biological function/pathway level, but not at the level of individual genes.

Conclusion

Chronic asymptomatic domoic acid exposure altered the transcription of genes in several IPA-identified Bio Functions related to nervous system function and neurodevelopment. The transcriptome response was highly dynamic over the 36 week exposure period, involving a varying list of differentially transcribed genes and revealing that gene transcription responses are unique at different exposure durations. Finally, oxidative stress-related mitochondrial dysfunction appears to play a role in chronic low-level exposure similar to that shown in previous acute exposure studies. These results highlight the temporal variability of the biological response to chronic perturbation of the glutamate system, and demonstrate the potential for subclinical pathology with low-level environmental toxin exposure.

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CHAPTER 3 – DOMOIC ACID DISRUPTS THE ACTIVITY AND CONNECTIVITY OF NEURONAL NETWORKS IN ORGANOTYPIC BRAIN SLICE CULTURES

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Abstract

Domoic acid is an algal-derived neurotoxin found in seafood during harmful algal blooms. As a glutamate agonist, domoic acid inappropriately stimulates excitatory activity in neurons; at high doses, this leads to seizures and brain lesions, but it is unclear how lower, asymptomatic exposures disrupt neuronal activity. Domoic acid has been detected in an increasing variety of species across a greater geographical range than ever before, making it critical to understand the potential health impacts of low-level exposure on vulnerable marine mammal and human populations. To determine whether prolonged domoic acid exposure altered neuronal activity in hippocampal networks, we used a state-of-the-art multi-electrode array (512-electrodes) with high spatial and temporal resolution to record extracellular potentials (spikes) in organotypic mouse-brain slice cultures. We identified individual neurons based on spike waveform and location, and measured the activity and functional connectivity within the neuronal networks of brain slice cultures. Domoic acid exposure significantly altered neuronal spiking activity patterns, and increased functional connectivity within exposed cultures, in the absence of overt cellular or neuronal death. While the overall spiking activity of neurons in domoic acid-exposed

cultures was comparable to controls, exposed neurons spiked significantly more often in bursts. We also identified a subset of neurons that were electrophysiologically silenced in exposed cultures, and putatively identified those neurons as fast-spiking inhibitory neurons. These results provide evidence that domoic acid affects neuronal activity in the absence of cytotoxicity, and suggest that neurodevelopmental exposure to domoic acid may alter neurological function in the absence of clinical symptoms.

Introduction

Domoic acid is an algae-produced seafood toxin that harms marine wildlife and humans by causing excitotoxicity in the central nervous system through over-stimulation of glutamate receptors (AMPA and kainate receptors) (Perl et al., 1990; Scholin et al., 2000; Stewart et al., 1990). Acute domoic acid exposure at high doses is well-known to cause seizures and neuronal death (Teitelbaum et al., 1990; L Tryphonas et al., 1990b), but the neurological impacts of lower-dose, sub-clinical exposure are not well-known. As domoic acid is becoming increasingly prevalent in the marine environment due to increases in harmful algal bloom frequency over an expanding geographical range (Anderson et al., 2012; Hallegraeff, 1993; Lefebvre et al., 2016; Moore et al., 2008), marine wildlife and humans are at heightened risk for domoic acid exposure. Recently, an unprecedented domoic acid-producing harmful algal bloom caused by a large warm-water anomaly persisted through all of summer 2015 along the Pacific coast of North America (NOAA, 2015); this bloom closed several important fisheries and severely poisoned hundreds of sea lions in California, Oregon, and Washington. California sea lions are regularly exposed to domoic acid

reaching mg/kg levels (Bejarano et al., 2007) during harmful algal blooms; these and other marine species likely face increasing exposure risk in the future as ocean temperatures continue to rise.

The developing fetus of pregnant mammals may be at particular risk because domoic acid crosses the placenta, enters the fetal brain, and is retained in amniotic fluid (Maucher and Ramsdell, 2007); neonatal domoic acid exposure can also occur through breast milk (Maucher and Ramsdell, 2007, 2005). This is especially concerning because domoic acid's disruption to the developing glutamate system, even at doses below those that cause seizures, can produce effects lasting into adulthood (Costa et al., 2010). For example, neonatal rats exposed to sub-clinical doses of domoic acid daily over postnatal days 8-14 exhibited a number of neurological and hippocampal alterations as adults, including increased axon sprouting, increased neurotrophic factor/receptor expression (Bernard et al., 2007; Doucette et al., 2004), reduction in a GABAergic subpopulation of neurons (Gill et al., 2010), novelty-induced seizures (Doucette et al., 2004), modified stress responses, increased perseveration, and altered search strategy (Gill et al., 2012). While these studies provide clear evidence of cellular/molecular and behavioral responses to developmental domoic acid exposure, there is little information about how neuronal activity and functional connectivity is affected, or how changes in hippocampal circuitry development may contribute to these reported functional deficits. Electrophysiological studies of neuronal activity can help bridge the findings of sub-clinical domoic acid exposure's cellular/molecular and behavioral impacts. For example, recording the activity of hundreds of neurons from within the same brain region can give insight into the networks these neurons form, how they communicate

with one another, and observe what changes arise from treatment with domoic acid. In contrast, recordings of individual neurons' activity can answer questions about a compound's affects on the function and physiology of a living cell, but that is difficult to extrapolate into effects on circuits of neurons that mediate regional function. Similarly, larger-scale imaging of activity in the functioning brain (e.g., functional MRI) lacks the resolution to reflect cellular details, leaving a gap in our ability to understand what happens in a population of individual neurons. Gaining a better understanding of how domoic acid influences the electrophysiological activity and connectivity of neural networks could provide insight into how cellular effects translate into behavioral outcomes.

We investigated the effect of sub-cytotoxic domoic acid exposure on the electrophysiological activity and connectivity of neural networks in organotypic hippocampal slice cultures using a state-of-the-art multi-electrode array that provided very fine spatial and temporal resolution (Litke et al., 2004). We hypothesized that domoic acid exposure, in the absence of overt cytotoxicity, would increase the activity and connectivity of neuronal networks due to elevated glutamate activity. Specifically, we quantified both neuron-level activity outcomes – including spike rate and burst rate – and network-level parameters (e.g., connectivity density) to address the knowledge gap between known domoic acid-induced cellular/molecular changes and changes in whole-brain function and behavior.

Methods

Organotypic brain slice cultures & domoic acid exposure

Cortico-hippocampal organotypic cultures (n=37) were prepared from seven mouse pups (age postnatal day 6; five-six slice cultures per brain) following the methods described by Stoppini et al. (1991). Briefly, brains were extracted, blocked into a cube (~5 mm per side) containing the hippocampus, and sectioned at 400 µm with a vibrating blade microtome (Leica VT1000 S). The sections were trimmed to include only the hippocampus and overlying cortex, and placed on circular filter paper (hydrophile membrane PTFE ~6 mm diameter, 0.4 µm pore size; BioCell Interface, Switzerland). Cultures were grown in culture media (1 mL per culture: 50% minimum essential medium (MEM), 25% horse serum, 25% Hank's balanced salt solution, 5 mg/mL D-glucose, 1 m*M* L-glutamine, and 5 U/mL penicillin-streptomycin) and maintained in an incubator for 16-17 days at 37°C and 5% CO_2 (see Ito et al. (2014) for more details). All animal care and treatments were approved by the institutional IACUC (UC Santa Cruz code: Litka1105) and adhered to NIH guidelines set forth in the Guide for the Care and Use of Laboratory Animals (NRC, 2011).

Half-volume (500 µL) culture media changes were done every 3 days, beginning the day after culture preparation (day 1 *in vitro*, DIV1). Cultures were randomly assigned to control (n=18) and domoic acid (n=19) treatment groups; assignments were balanced evenly across cultures that originated from the same mouse. Exposure to 0.1 µ*M* domoic acid began on DIV4, using culture media containing domoic acid, and continued through DIV16-17.

This domoic acid dose was selected to be below those shown to cause overt cytotoxicity: our dose is 10–15-fold lower than the ≥1 to 5 µ*M* levels shown to cause

cell damage/death in the CA1, CA3 and dentate gyrus in hippocampal brain slice cultures (Pérez-Gómez and Tasker, 2012). Domoic acid concentrations in culture media aliquots collected prior to and following organotypic culture exposures were determined by ELISA (Biosense Laboratories, Bergen, Norway), and were not measurably different from the expected target dose (data not shown).

Multi-electrode array recording & spike-sorting

Electrophysiological activity from brain slice cultures (n=14 control, n=15 domoic acid) was recorded on DIV16 or 17 using a custom-made 512-electrode array system (Litke et al., 2004). This array features flat electrodes 5 µm in diameter and spaced 60 µm apart in a hexagonal lattice over a 0.9 mm x 1.9 mm rectangular area. Cultured brain tissues and adherent filter paper were gently placed on the electrode array, tissue-side down, with the hippocampal region centered on the array. A small, circular weight (~1.3 g) with fine mesh (160 mm pore size) was placed on the filter paper on top of the tissue to maintain even contact between the tissue and the array. The recording chamber (~1.8 mL volume) housing the array and mounted tissue was filled with fresh culture media (no domoic acid), and was kept perfused with culture medium (95% O_2 / 5% CO_2, 37°C) at a flow rate of 3 mL/min.

Prior to electrophysiological recording, tissues were equilibrated in the recording chamber for 30 min to avoid any transients that could have been caused by temperature differences from moving the culture from the incubator to the chamber. Extracellular signals from neuronal action potentials (spikes) were recorded for 90 minutes on each of the 512 electrode channels at a sampling rate of 20 kHz (i.e., 50 µsec temporal resolution). Raw waveforms were then spike-sorted using methods developed by Litke et al. (2004). Briefly, spike-sorting uses the electrophysiological

activity recorded by each electrode to identify individual neurons based on the timing, waveform, and location of spiking activity on the array (see Supplemental Information for more details). This method significantly extends the information gained by simply assessing electrophysiological activity at each electrode, because more than one neuron may be contributing to the activity detected by a single electrode. Our spike-sorting analyses allowed us to evaluate individual neurons' electrophysiological activity.

Identification of hippocampal neurons in electrophysiological recordings

In general, the hippocampi of mouse organotypic brain slice cultures were slightly smaller than the array, covering ~70-80% of the recording area (Fig S1). Because some activity from cortical neurons was occasionally included at the margins of the array, we identified and selected hippocampal neurons for analysis. This was accomplished using a photographic overlay process, in which we compared brightfield photomicrographs of the cultures taken on DIV1, which exhibits the clearest visual tissue morphology, and again after recording on DIV 16 or 17, including the 512-array. The map of identified neurons from each recording, determined through spike-sorting analyses (section 2.2), were plotted on top of the corresponding tissue's DIV1 photograph to visualize with the underlying anatomical structures. Only electrophysiologically active neurons within the hippocampal region were retained for further analysis of the hippocampal network structure (see Ito et al. (2014) for additional detail).

Neuronal spike rate and bursting activity assessment

Spike rate was defined as the number of spikes (action potentials) a neuron produces per second across the 90-minute electrophysiological recording, while burst rate was defined as the number of bursts a neuron produces per minute over the 90-minute recording. To calculate how often hippocampal neurons spiked in bursts, we used a modified Poisson surprise algorithm (Stafford et al., 2009). This method uses the mean inter-spike interval (the average time between each action potential) of a neuron to define the criteria that a particular neuron needs to meet for its spikes to qualify as a burst. When the inter-spike interval for a series of 3+ spikes was less than half the mean inter-spike interval, and the probability that such an event occurred by chance was less than 10^{-4}, then that cluster of spikes was defined as a burst. Thus, burst rate (bursts/min) measured the degree to which a neuron spiked exclusively in temporally concentrated clusters of spikes. That is, a neuron with a high burst rate spiked in distinct temporal bands, with inactivity in between each "burst"; a neuron with a low burst rate spiked at regular intervals, at any spike rate, and did not have distinct, alternating periods of activity/inactivity (see Supplemental Information for more details).

Effective connectivity density measurement by transfer entropy analysis

Connectivity density was defined as the percentage of connected neuron pairs out of the total number of possible neuron pairs on the array. We used transfer entropy to calculate the effective connectivity density – as opposed to physical connections – within the neural networks of recorded tissues. Physical connections, such as synapses between neurons, are not always electrophysiologically active (i.e., "silent" synapses), and not all neurons exhibit spontaneous electrophysiological activity in

organotypic brain slice cultures; effective connectivity instead describes information flow and correlated activity patterns between active neurons. Transfer entropy is an information-theoretic calculation that identifies interactions between two neurons based on the timing of their spikes (action potentials) (Ito et al., 2011). In this calculation, a neuron pair is identified as connected when the spike history of neuron 1 improves the ability to predict the spiking activity of neuron 2 beyond the prediction based on neuron 2's spike history alone. That is, when two neurons spike in a temporally synchronous pattern, the transfer entropy calculation will be more positive than a neuron pair that does not spike synchronously (Shimono and Beggs, 2014). Equations and algorithm details/parameters can be found in the Supplemental Information. Also see Ito et al. (2011), Shimono & Beggs (2014) & Timme et al. (2014) for more thorough discussion of transfer entropy and its applications in analyzing the neural networks of organotypic brain slice cultures.

Immunohistochemistry & imaging

Immediately after electrophysiological recording, photographs of all organotypic cultures on the 512-electrode array were taken using a Nikon CoolPix 995 camera mounted on a Leica DM IL inverted microscope to record the exact location of the tissue on the microelectrode array. Immediately following imaging, the cultures were gently lifted from the array and fixed in 4% paraformaldehyde for 20 minutes, then stored in phosphate buffered saline with 0.05% sodium azide at 4°C. The eight non-recorded cultures were also fixed/stored in the same manner on DIV17.

For NeuN immunostaining, brain slice cultures (n=10 control: 6 recorded, 4 non-recorded; n=12 domoic acid: 8 recorded, 4 non-recorded) were processed free-floating. Antibodies used were: mouse monoclonal IgG1 anti-NeuN (neuronal nuclei

– Chemicon Millipore (cat #MAB377), 1:1000); goat anti-mouse IgG (Molecular Probes Alexa Fluor 555, 1:1000). A 10-min DAPI incubation (Invitrogen D21490/DAPI-Fluoro- Pure Grade, 300 n*M* working solution) was included to stain for cell nuclei. Cultures were mounted, coverslipped with Fluoromount-G (Southern Biotech), and allowed to dry overnight before imaging. (See Supplement for full protocol.)

For staining-based cell counts, cultures were imaged on a Keyence Biorevo BZ-9000 digital widefield microscope using a 40x/0.95 objective lens; exposure time was optimized to the z-plane of highest-intensity staining for each field of view in each culture. All other scope and imaging parameters were kept constant. One z-stack (0.6 µm spacing) per channel (both DAPI and NeuN) was captured within each hippocampal sub-region (CA1, CA3, and DG) per culture.

Cell counts

Cell counts were performed on the z-stack images taken at 40x magnification using the Surfaces algorithm of Imaris software (BitPlane, Concord, MA). We used both voxel intensity and size thresholds for NeuN (intensity threshold = 10; size thresholds = ≥5 µm, ≥2000 voxels) and DAPI (intensity threshold = 35; size thresholds = ≥7 µm, ≥2000 voxels) labeling. The number of surfaces detected by the algorithm yielded the number of total cells ($DAPI^+$) and neurons ($NeuN^+$). For a subset of NeuN images (18 of 66 images: 5 of 30 control, 14 of 36 domoic acid), background and non-specific staining were present to a degree that the thresholds set in the surface algorithm were unable to successfully filter out cellular debris; the total number of surfaces detected by Imaris was erroneously high. For those images, a cell-counting module in ImageJ/FIJI (NIH) was used to count neurons by hand.

Statistical analyses

All data are given as means ± standard error of the mean (SE) or least squares mean ± standard error, as indicated. For cell and neuron counts (both immunostained and electrophysiologically active) and connectivity density, a mixed model ANOVA was used to assess differences between control and domoic acid-exposed cultures, with treatment as a fixed effect and the animal each culture originated from a random effect (due to a significant interaction between treatment and animal). For neuron-level outcomes (spike rate & burst rate), the random effect was modified so that the organotypic culture that each neuron was measured in was nested within the animal that the culture was generated from. For the percent bursting and percent putative inhibitory neuron outcomes, a binomial regression was used to assess the differences in cell-type proportions within control and domoic acid-exposed cultures. Data for NeuN count, spike rate, and burst rate were log-transformed, and connectivity density was logit transformed to achieve Gaussian distribution prior to analyses. JMP Pro 12.0.1 (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses. Statistical significance is indicated as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

Results & Discussion

Domoic acid exposure was not overtly cytotoxic

We used a domoic acid exposure level (0.1 μ*M*) that was <10% of levels (≥1-5 μ*M*) that have been shown to cause neuronal death in brain slice cultures (Pérez-Gómez and Tasker, 2012). To confirm that our low-dose domoic acid exposure paradigm did not cause overt cell or neuronal death, we conducted total cell and neuron counts in the hippocampi of organotypic brain slice cultures using DAPI and NeuN immunohistochemical staining. Results show that domoic acid exposure had no measurable effect on either total cell numbers ($DAPI^+$ in the CA1, $F(1,15.8) = 0.12$, $p = 0.74$; CA3, $F(1,13.2) = 0.65$, $p = 0.43$; and dentate gyrus (DG), $F(1,16.7) = 0.50$, $p = 0.49$) or the number of neurons ($NeuN^+$ cells in the CA1, $F(1,14.9) = 0.08$, $p = 0.79$; CA3, $F(1,14.6) = 2.07$, $p = 0.17$; and DG, $F(1,12.0) = 0.08$, $p = 0.78$) (Fig. 1D and 1E). The proportion of neurons to total cells within each field of view was also not affected (data not shown).

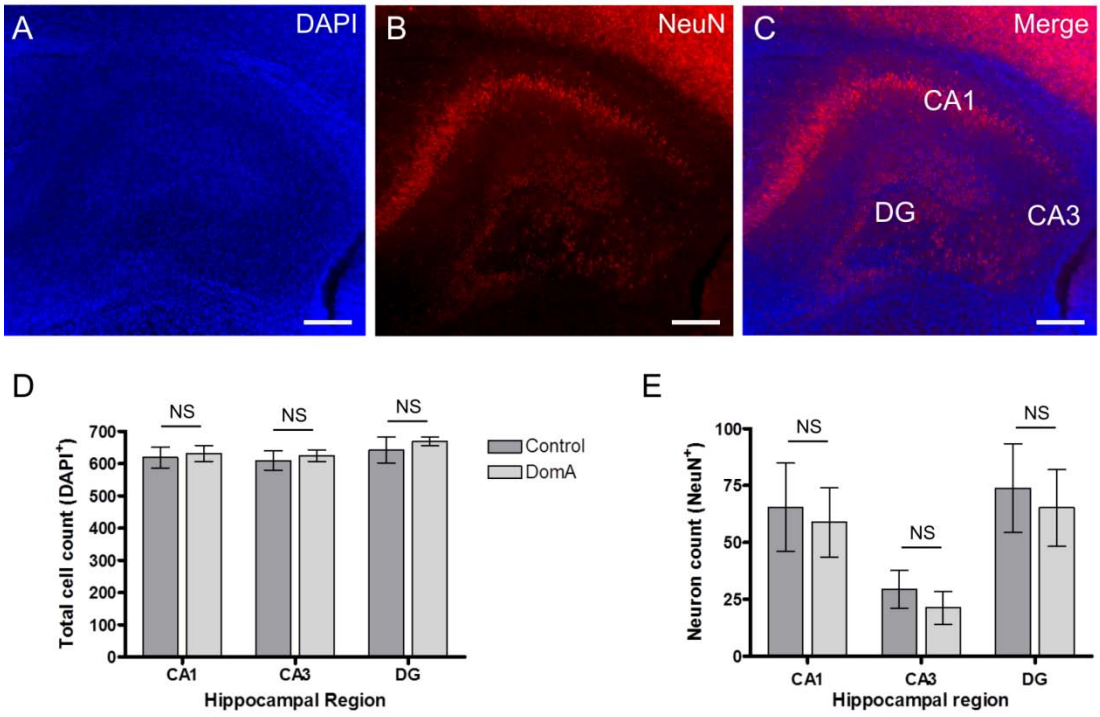

Figure 1. Domoic acid exposure had no effect on the number of total cells or neurons. Immunostaining and quantification of neurons ($NeuN^+$) and total cells ($DAPI^+$) in the hippocampi of organotypic brain slice cultures. (A-C) Representative organotypic brain slice culture (brain slice ID 6-6; x4 magnification) stained with DAPI (A) (blue; labels all cell nuclei) and NeuN (B) (red; labels neuronal nuclei), and merge (C), with hippocampal subregions CA1, CA3, dentate gyrus (DG) indicated. Scale bar = 200 µm. Quantification of total cell numbers (D) and neuron counts (E) per hippocampal subregion for both control (n=10, mean ± SE) and domoic acid-exposed (n=12) tissue cultures. NS = not significantly different, $p > 0.05$ for all (mixed model ANOVA).

Domoic acid exposure reduced the number of electrophysiologically active neurons

In order to determine whether the number of electrophysiologically active neurons in the hippocampus was affected by domoic acid exposure, we identified all active neurons by spike-sorting the action potentials recorded by the 512-array (see Methods) and specifically selected neurons within the hippocampus (Fig. 2A). We found that domoic acid-exposed brain slice cultures had significantly fewer active

hippocampal neurons than control cultures (Fig. 2B, Table S1) (control 134 ± 21.9 neurons (least squares mean ± SE), domoic acid 93.8 ± 21.6 neurons; $F(1,22.5) = 6.76$, $p = 0.016$). Taken with the previous finding that the total number of neurons was not affected (Fig. 1), this suggests that a subset of neurons were electrophysiologically silenced following prolonged *in vitro* low level domoic acid exposure. Note that although the number of active hippocampal neurons should be a subset of the number of hippocampal neurons in the brain slice culture measured by immunostaining, those two datasets cannot be directly compared because the three fields of view for microscopy analyses does not correspond to the area measured by the 512-electrode array.

This reduction in the number of active neurons may be attributed to the presence of "silent" synapses in the domoic acid-exposed cultures. Physical synapses may be functionally silent and non-responsive to synaptic transmission through removal of AMPA receptors from the synaptic membrane (Isaac et al., 1995; Liao et al., 1995). AMPA receptors are a specific sub-type of glutamate receptor that domoic acid binds to with strong affinity (i.e., 9 n*M*) (Carcache et al., 2003; Hampson et al., 1992). Persistent, weak synaptic stimulation – perhaps similar to that caused by prolonged low dose domoic acid exposure – has been shown to cause a weakening of functional synaptic connections due to internalization of AMPA receptors from the neuron membrane (Czöndör and Thoumine, 2013; Hanse et al., 2013). AMPA receptor internalization is accomplished through dephosphorylation of the receptor; interestingly, domoic acid exposure (400 n*M* for 1 hour) has been shown to decrease AMPA receptor phosphorylation in rat hippocampal slice cultures (Qiu et al., 2009). While our data does not directly prove or identify a specific mechanism for neuron

silencing, it is plausible that neurons responded to prolonged domoic acid exposure by altering their activity or excitability to compensate for the excitatory environment in which they were cultured. After removal from the domoic acid-contaminated media, any persistent compensatory changes in the neuronal activity would be measured by the 512-array during recording.

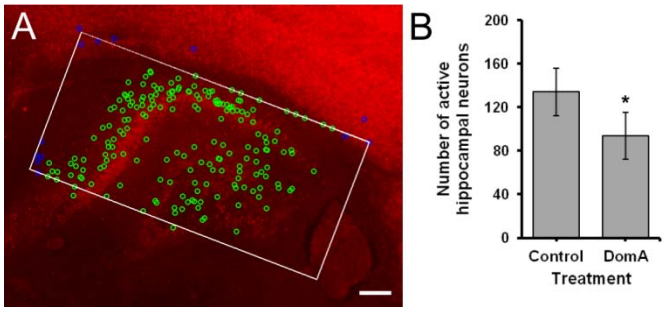

Figure 2. Number of electrophysiologically active neurons in the hippocampi of brain slice cultures is reduced by domoic acid exposure. (A) Photomicrograph (x4 magnification; scale bar = 200 µm) of a representative brain slice culture (red staining = $NeuN^+$ neurons). White box highlights the region covered by the 512-electrode array, and electrophysiologically identified neurons (circles) are overlaid. Green circles = hippocampal neurons; blue circles = cortical neurons (excluded from analyses). (B) Number of electrophysiologically active hippocampal neurons (least squares mean ± SE) in control (n=14) and domoic acid-exposed (n=15) brain slice cultures. * $p < 0.05$ (mixed model ANOVA; note that the LS errors from the model do not fully reflect the contrast or significance between control and domoic acid treatment detected by the model).

Domoic acid exposure did not significantly alter neuronal spike rate, but significantly increased neuronal bursting activity

In order to broadly assess how domoic acid exposure altered electrophysiological activity patterns in the neural networks of brain slice cultures, we visually examined neural activity profiles and raster plots. Neuron activity profiles (Fig. 3A, B) show the average spiking rate of all hippocampal neurons over the 90-minute duration of the

recording. For example, in brain slice culture ID 3-1 (Fig. 3A), 104 hippocampal neurons were identified through spike-sorting (see Methods), and the spike rates of all neurons within a 1-second time period were averaged for each of the 5400 seconds (90 min) of electrophysiological recording (x-axis). The representative activity profiles shown provide qualitative evidence of alterations caused by domoic acid exposure – for example, more large-amplitude spike rates (arrowheads) and lower baseline activity (arrows) in the domoic acid exposed culture (see Fig. S3 for all activity profiles). Raster plots (Fig. 3C,D) illustrate individual neurons' spikes over time. Qualitative visual assessment of raster plots revealed that many fast-spiking neurons (arrows in Fig. 3C,D) are absent in the domoic acid-exposed brain slice cultures compared to controls (Fig. S4). These differences suggest that domoic acid exposure altered activity patterns in the neural networks of organotypic brain slice cultures.

In order to quantify the observed changes in electrophysiological activity, we calculated both the spike rate (spikes/sec: Hz) and burst rate (bursts/min) of hippocampal neurons in all control and domoic acid-exposed brain slice cultures. Spike rate was calculated on a per-neuron basis by dividing each neuron's total number of recorded spikes by the recording duration (5400 sec). Burst rate (i.e., the number of bursts a neuron produces per minute) was calculated with a modified Poisson surprise algorithm (see Methods). This algorithm identified bursts based on the degree to which a neuron spiked only in temporally concentrated clusters: A neuron with a high burst rate spiked in distinct temporal bands, with inactivity in between each burst; a neuron with a low burst rate spiked at regular intervals, at any spike rate, and did not have distinct periods of alternating activity/inactivity.

We found no difference in the mean spike rates (Hz) of active neurons in domoic acid-exposed cultures compared to neurons in control cultures (Fig. 4, Table S1) (control 0.32 ± 0.02 Hz (least squares mean ± SE), domoic acid 0.29 ± 0.02 Hz; $F(1,24.6) = 1.46$, $p = 0.24$). However, there was a significant increase in the proportion of bursting vs non-bursting neurons in domoic acid-exposed vs control cultures (Fig 5A, Table S1) (control 91.6 ± 3.1%, domoic acid 96.8 ± 1.2%; $p = 0.0041$). The burst rate of bursting neurons was also significantly increased in domoic acid-exposed cultures compared to neurons in control cultures (Fig. 5B, Table S1) (control 0.43 ± 0.04 min^{-1} (least squares mean ± SE), domoic acid 0.67 ± 0.07 min^{-1}; $F(1,24.5) = 8.80$, $p = 0.0066$).

In other studies, short-term domoic acid exposure (~10 minutes at 0.5-2 µ*M*; ~30-60 minutes at ~3-1000 µ*M*) reduced the mean spike rate (spikes/sec) in primary mixed neuronal/glial cortical cell cultures in a dose-dependent manner (Hogberg et al., 2011; Mack et al., 2014; Wallace et al., 2015). Mean burst rate (bursts/min, where a burst is defined as ≥5 spikes in 100 msec) was also reduced by short-term/acute exposure to domoic acid (~10 minutes at 0.5-2 µ*M*) (Hogberg et al., 2011). Interestingly, in the same mixed cell culture model, prolonged exposure (28-35 days *in vitro*) to 50 n*M* domoic acid actually *increased* the mean spike rate and mean burst rate (Hogberg et al., 2011). Notably, for all of these recordings domoic acid was present in the culture medium, suggesting that the reported increases in spike- and burst rate are due to the excitatory effect of domoic acid on neuron activity during electrophysiological recording.

In our study, brain slice cultures were washed with domoic acid-free medium for 30 minutes prior to recording, and were recorded in domoic acid-free medium. This

allowed us to measure the persistent effects of domoic acid exposure on neuronal activity in the brain slice cultures caused by prolonged exposure and growth/development in an excitatory environment. Our findings that domoic acid exposure increased neuronal burst rate (bursts/min) without affecting mean spike rate (Hz) suggests that the pattern of neuronal activity was altered in the domoic acid-exposed cultures, but not the overall activity as reflected by the mean spike rate. In the absence of changes in total neuronal network activity, it is possible that altered activity patterns may affect how neurons interact with one another within the network.

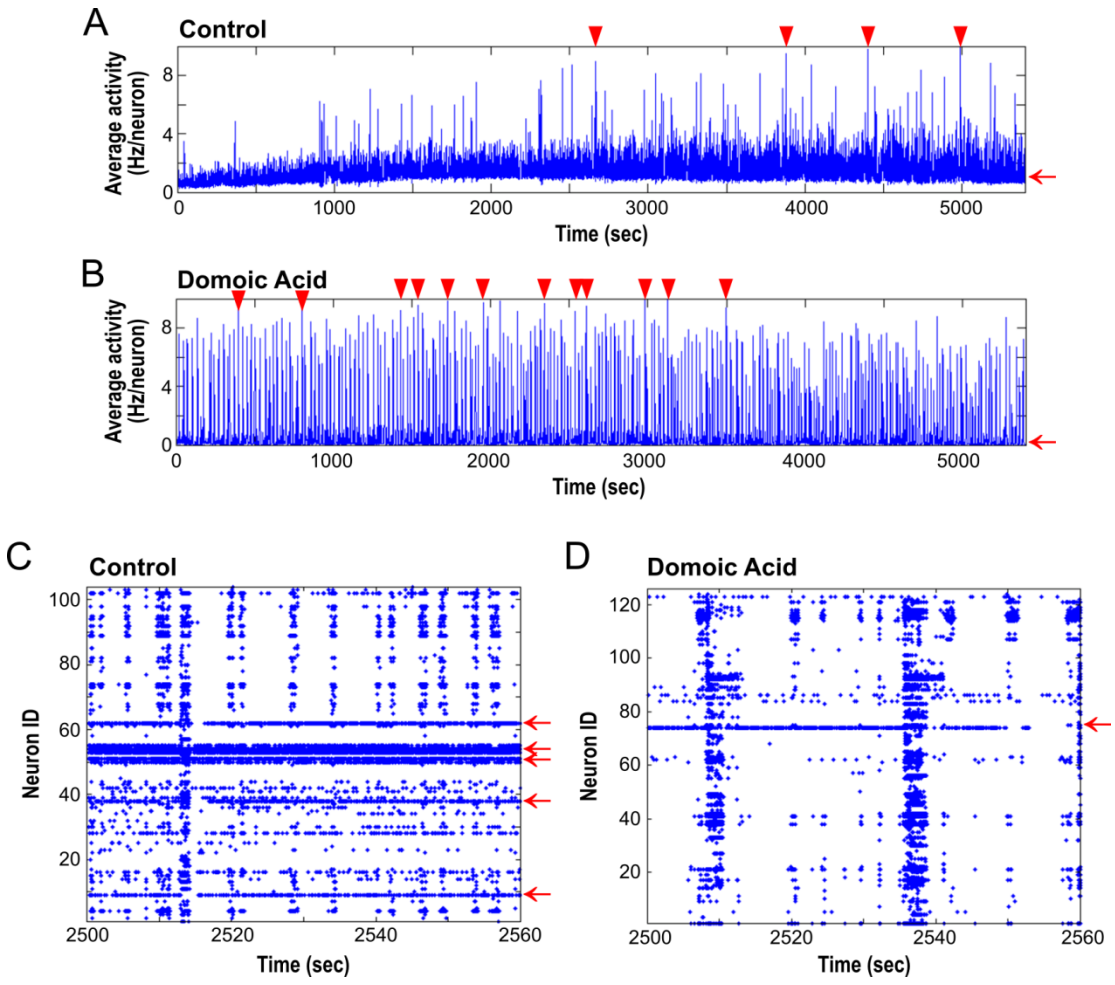

Figure 3. Domoic acid exposure altered the pattern of electrophysiological activity in brain slice cultures. Electrophysiological activity profiles for representative control and domoic acid-exposed brain slice cultures. (A & B) Activity traces show average electrophysiological activity (Hz/neuron) of representative (A) control (tissue ID 3-1; 104 active neurons) and (B) domoic acid-exposed (tissue ID 3-2; 126 active neurons) brain slice cultures over the duration of the recording (90 min). (C & D) Raster plots for a representative one-minute interval of the recordings shown in A & B illustrate spike activity on a per-neuron basis. Individual dots (•) indicate one action potential (spike) for each neuron, with neuron ID along the y-axis. Note the decrease in the number of fast-spiking neurons (arrows) between the control (C) and domoic acid-exposed (D) tissues.

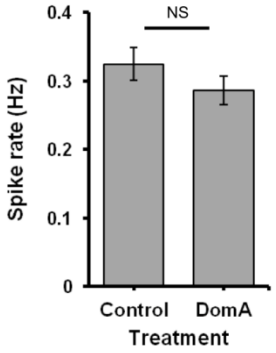

Figure 4. Spike rate (Hz) of active neurons in brain slice cultures was not affected by domoic acid exposure. Spike rate of hippocampal neurons in control (n=14; n=23-264 neurons per culture) and domoic acid-exposed (n=15; n=22-206 neurons per culture) brain slice cultures. NS = not significantly different. *p*=0.24 (mixed model ANOVA).

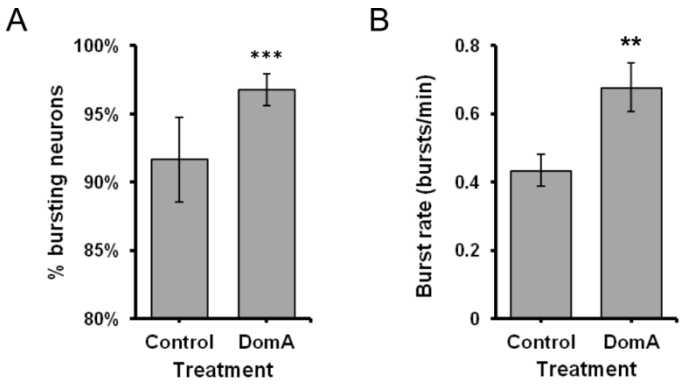

Figure 5. Domoic acid exposure increased the burst rate (bursts/min) of active neurons in brain slice cultures. (A) Percentage of hippocampal neurons exhibiting bursting activity (mean ± SE) within control (n=14) and domoic acid-exposed (n=15) cultures. *** $p < 0.005$ (binomial regression). (B) Burst rate (bursts/min; mean ± SE per culture) of hippocampal neurons in control (n=14 cultures; n=14-254 neurons per culture) and domoic acid-exposed (n=15 cultures; n=20-203 neurons per culture) brain slice cultures. ** $p < 0.01$ (mixed model ANOVA).

Domoic acid increased the functional connectivity density in electrophysiologically active neurons

In order to quantify changes in functional connectivity within the neural networks of organotypic brain slice cultures, we calculated the 'connectivity density' within each culture. Connectivity density is defined as the percentage of hippocampal neuron pairs identified as connected out of all possible active neuron pairs. Whether or not a neuron pair was connected was determined using transfer entropy, an information theoretic model that identifies functional neuronal connections based on both spike timing and coordination with other active neurons (Ito et al., 2011; Shimono & Beggs, 2014; see Methods). Connectivity density measures functional connectivity, as opposed to physical connectivity; that is, it provides information on the electrophysiological activity within the neural network, rather than a count of physical synapses (Ito et al., 2011).

We found that domoic acid exposure increased connectivity density from 1.39% (± 0.24%) in control cultures to 2.73% (± 0.50%) in domoic acid-exposed cultures ($F(1,22.7) = 8.61$, $p = 0.0075$) (Fig. 6). Increased functional connectivity may be consistent with changes in physical connectivity that have been shown with domoic acid exposure in previous studies. For example, increased mossy fiber sprouting, a form of abnormal synaptic reorganization within the dentate gyrus of the hippocampus, has been documented in domoic acid-exposed brain slice cultures (Pérez-Gómez and Tasker, 2013), domoic acid-exposed sea lions (Buckmaster et al., 2014), and lab rats exposed to domoic acid in the early pre-weaning lifestage (Bernard et al., 2007). Enhanced synapse formation, as measured by the pre- and post-synaptic markers synaptophysin and PSD-95, respectively, has also been

reported in brain slice cultures in response to domoic acid exposure (2 µ*M* for 24 hours) (Pérez-Gómez and Tasker, 2013).

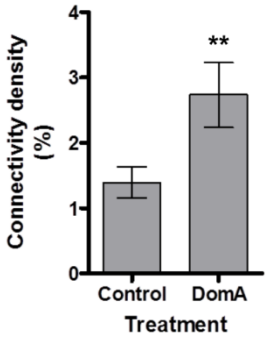

Figure 6. Domoic acid exposure increased connectivity density within the neural networks of brain slice cultures. Connectivity density (mean ± SE) within the hippocampal networks of control (n=14) and domoic acid-exposed (n=15) brain slice cultures. Connectivity density is the percentage of all possible active neuron pairs that are functionally connected as determined by their coordinated spiking activity. ***p* < 0.01 (mixed model ANOVA).

Domoic acid exposure caused the silencing or loss of a subset of fast-spiking neurons

In order to quantify the silencing/loss of fast-spiking neurons in the domoic acid-exposed brain slice cultures (red arrows, Fig. 3C versus 3D, section 3.3), we explored whether these neurons exhibited a distinct electrophysiological activity phenotype based on their spike rate (Hz) and burst rate (bursts/min). As these neurons appeared to be continuously spiking at regular intervals (i.e., high spike rate with low burst rate), we set a "high spike rate" criteria of ≥5 Hz and a "low burst rate" criteria of ≤1 burst/min (see insets in Fig. 7A,B). Plotting individual neurons' spike rate versus burst rate (Fig. 7A,B) in control and domoic acid-exposed cultures showed that the proportion of fast-spiking neurons (proportion of total active

neurons) was significantly reduced in domoic acid-exposed brain slice cultures compared to controls (control: 4.16% ± 0.68% of total neurons, domoic acid: 0.87% ± 0.23%; $p = 0.0002$) (Fig. 7C, Table S1).

To further characterize this subset of neurons, we examined the nature of their functional connections with other neurons. For this, we produced cross-correlation histogram plots for each set of functional neuron pairs to reflect the correlated spike-timing relationship between the connected neuron pairs (Ito et al., 2014). We qualitatively assessed the cross-correlation histograms for the 86 fast-spiking neurons (74 control, 12 domoic acid) identified in control and domoic acid-exposed cultures to observe whether there was a clear pattern of connection (i.e., inhibitory trough or excitatory peak to the right of zero; see Fig. 7D for example cross-correlation histogram). Of the identified fast-spiking neurons, 64 had observable connections (75 total connections of 53 fast-spiking neurons in control cultures, and 19 connections of 11 fast-spiking neurons in domoic acid-exposed cultures). We found that 35 neurons exhibited inhibitory connections (28 control, 7 domoic acid), 2 neurons exhibited excitatory connections (1 control, 1 domoic acid), and the remainder did not exhibit clear patterns of connectivity (i.e., lacked a trough or peak). Based on this, we putatively identified the fast-spiking neurons as inhibitory. Our conclusion that these 'putative inhibitory' (PI) neurons are silenced/lost is consistent with evidence that domoic acid exposure reduces inhibitory activity (as measured by inhibitory post-synaptic potentials) in brain slice cultures following exposure to high-affinity kainite receptor agonists, including domoic acid (1 μ*M*) (Clarke and Collingridge, 2004).

One intriguing possibility is that these silenced/lost neurons are fast-spiking/parvalbumin^{+} interneurons. These are a subset of GABAergic neurons that spike very rapidly and help regulate excitatory activity in the hippocampus; they play important roles in basic microcircuit functions and complex network operations (Hu et al., 2014). Fast-spiking/parvalbumin^{+} interneurons comprise ~2.6% of hippocampal neurons (Hu et al., 2014) and ~5% of cortical neurons (Behrens and Sejnowski, 2009), which corresponds closely to our measured 4.2% PI neurons in control cultures. And although we cannot determine the mechanism through which neurons were silenced/lost with domoic acid treatment, GABAergic neurons are known to express AMPA receptors (He et al., 1998; Racca et al., 1996), and may have exhibited a compensatory response to the prolonged domoic acid treatment by internalizing them. Inhibitory neurons normally maintain a high proportion of silent synapses, regardless of developmental period/window (Hanse et al., 2013), and are highly sensitive to changes in glutamatergic activity (He and Bausch, 2014). As a potent glutamate agonist, domoic acid could have readily affected the synapses and/or activity of these neurons. The slow development of parvalbumin^{+} neurons also increases their vulnerability to insults that can permanently affect their maturation and function (Behrens and Sejnowski, 2009), which potentially makes them especially sensitive to domoic acid exposure during neurodevelopment. Domoic acid-induced disruptions to the activity of this critical sub-population of neurons, even in the absence of seizures and cell death, would likely have consequences lasting into adulthood and potential impacts on normal brain function.

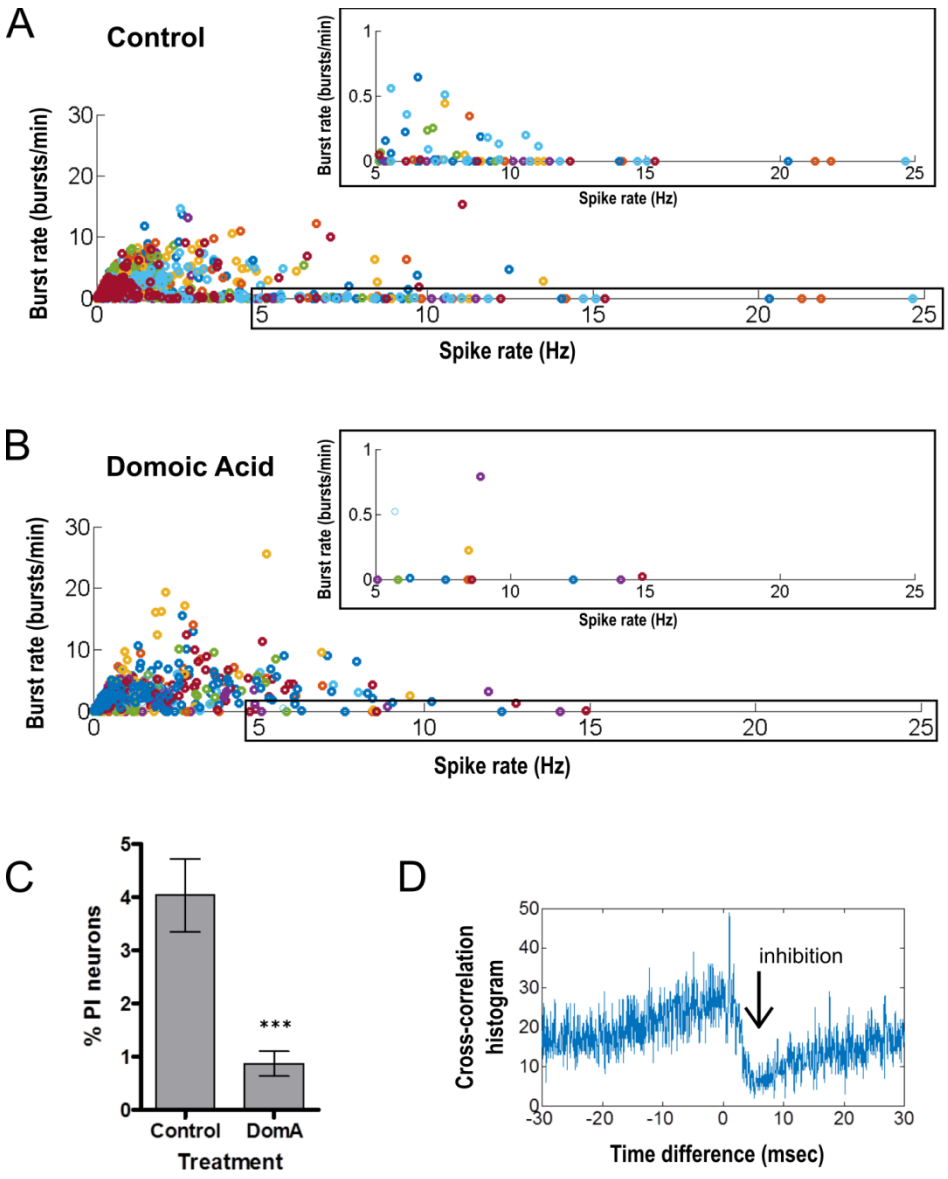

Figure 7. Identification, quantification, and characterization of putative inhibitory neurons silenced in domoic acid-exposed brain slice cultures. (A, B) Constantly spiking neurons, characterized by a high spike rate (Hz) and low burst rate (bursts/min) (expanded in insets) are missing from domoic acid-exposed tissue cultures. All neurons from all recorded cultures are included; color corresponds to individual cultures. (C) Quantification of the fraction of these putatively inhibitory (PI) neurons within control (n=14) and domoic acid-exposed (n=15) cultures. ***$p < 0.0005$ (binomial regression). (D) Cross-correlation histogram for a representative PI neuron; cross-correlation histograms illustrate the relationship of one neuron's spiking activity with another's. For example, if neuron A has an excitatory effect on neuron B, whenever neuron A spikes, neuron B will preferentially spike immediately afterward, and there will be a peak in the

cross-correlation histogram just to the right of zero. Alternatively, if neuron A has an inhibitory effect on neuron B, there will be a trough just to the right of zero. In this plot, the depression after the 0 msec time difference (arrow) indicates that this PI neuron suppresses spiking activity in the second neuron.

Conclusions

Prolonged sub-clinical domoic acid exposure altered the activity pattern and connectivity density of neural networks in organotypic brain slice cultures, and silenced a subset of fast-spiking neurons (putatively identified as inhibitory neurons). These kinds of electrophysiological alterations have been linked to neurological dysfunction *in vivo*, which underscores the potential for neurodevelopmental domoic acid exposure to cause lasting effects on neurological function. The novel array technology we utilized, accompanied by powerful computational tools, provided the ability to investigate neural network activity and function with single-neuron specificity. This kind of detailed electrophysiological study can help bridge findings of the effects of sub-clinical domoic acid exposure on cellular/molecular processes with behavioral impacts. Our collaborators at the Santa Cruz Institute for Particle Physics have also recently developed an array system to record hundreds of neurons *in vivo* in the brains of awake, behaving mice, providing direct access to hundreds of neurons and the networks they form.

Acknowledgements

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SUPPLEMENTAL MATERIALS

Methods

Spike-sorting: Extracellular signals (i.e., changes in extracellular potential) measured by the 512-array that crossed a threshold of 8 standard deviations of background noise were identified and selected. The waveforms detected by a specific electrode and its six adjacent neighbors were projected into five dimensional principal component space. A mixture of Gaussians model was fit to the distribution of features based on an expectation maximization algorithm. Duplicate neurons, neurons with refractory period violations, and neurons with low activity (<100 spikes/hour) were excluded from further analysis.

Bursting activity assessment: Bursting activity was assessed using an approach modified from Stafford et al. (2009) (equation 1), where P_{Ci} is the initial probability that a neuron with mean spike rate λ would have C_i spikes in a time bin T; T is the time between the first and last spikes in question. Spikes are added to the burst until the P_{Ci} including the newest spike is greater than the initial value, or the next spike occurs longer than the mean inter-spike interval (ISI) after the preceding spike. A burst occurs when the maximum $P_{Ci} < 10^{-4}$.

$$P_{C_i} = e^{-(\lambda \times T)} \times \frac{(\lambda \times T)^{C_i}}{C_i!} \quad (1)$$

Connectivity density and transfer entropy analyses: We followed a method described in Shimono & Beggs (2014) for testing the significance of identified connections between neurons. This was done using two parameters: transfer entropy (TE) and

the coincidence index (CI). Transfer entropy (equation 2) examines the relationship between two neurons' spiking history, and yields an increasingly positive number when neuron *J*'s spiking history improves the ability to predict neuron *I*'s spiking activity beyond neuron *I*'s spiking history alone. The time bin of neuron *J* was delayed by *d* bins (equation 3) to account for natural differences in synaptic delays between neurons. Plotting the transfer entropy as a function of that delay (*d*) produces a graph similar to Figure S2; the peak of that function is the value that represents effective/functional connectivity between the two neurons.

Because TE identifies both individual connections and network bursts (a majority of neurons firing simultaneously), we needed a secondary measure to remove connections produced by network bursts and keep neuron-neuron interactions. The coincidence index (CI; equation 4, Fig S2) measures the tendency for TE values to peak sharply (neuron-neuron pair) or broadly (network burst). To establish whether a neuron pair's connection was significant, both the TE and CI values had to be significantly larger than if the TE and CI values for that neuron pair had been produced by chance (accomplished by jittering the pre-connection neuron (neuron *I*) data by 15 msec; see Shimono & Beggs (2014) for further detail).

$$TE_{J\to I} = \sum p(i_{t+1},\, i_t^{(k)},\, j_t^{(l)}) \log_2 \frac{p(i_{t+1}|i_t^{(k)},\, j_t^{(l)})}{p(i_{t+1}|i_t^{(k)})} \qquad (2)$$

i_t = status of neuron *I* at time *t*; can be either 1 or 0, indicating a spike or no spike, respectively

j_t = status of neuron *J* at time *t*

i_{t+1} = status of neuron *I* at time *t+1*

p = probability of having the status in the following parentheses

| = conditional probability

Σ is over all possible combinations of i_{t+1}, $i_t^{(k)}$, and $j_t^{(k)}$

k & *l* = order of TE; the number of time bins in the past that were used to calculate the histories of systems *I* and *J*, respectively (Here, *k* = *l* = 1; only single time bins considered)

$$TE_{J\to I}(d) = \sum p(i_t,\ i_{t-1},\ j_{t-d}) \log_2 \frac{p(i_t|i_{t-1}, i_{t-d})}{p(i_t|i_{t-1})} \quad (3)$$

d = different time delays (1-15 msec)

$$CI = \frac{\sum_{d=0}^{d=\tau} TE(d)}{\sum_{d=0}^{d=T} TE(d)} \quad (4)$$

TE(d) = transfer entropy measured at delay *d*

τ = coincidence window size (3 msec)

T = entire window size of the measure (15 msec)

Immunostaining: Cultures (n=10 control: 6 recorded, 4 non-recorded; n=12 domoic acid 8 recorded, 4 non-recorded) were processed for immunostaining free-floating, ensuring that the tissue side faced upward (filter paper down) at all times. All steps were performed on an orbital shaker table (Heidolph™). After being washed three times with tris-buffered saline (TBS), cultures were blocked with 10% normal goat serum and permeabilized with 0.5% Triton-X for 2 hours at room temperature. Following another three washes with TBS, cultures were incubated with primary antibody (Neuronal nuclei (NeuN) – Chemicon Millipore (cat #MAB377) mouse monoclonal IgG1 anti-NeuN, 1:1000) overnight at 4°C.

Cultures were then washed 3x with TBS and incubated with secondary antibody (Molecular Probes Alexa Fluor 555 goat anti-mouse IgG, 1:1000) for 2 hours at room temperature. Next, cultures were washed 3x with TBS, DAPI stained for 10 min

(Invitrogen D21490/DAPI-Fluoro- Pure Grade, 300 n*M* working solution), and washed 3x with TBS. Tissues were mounted on superfrost/Plus slides (Fisherbrand), then coverslipped (Slip-Rite #1.5 coverglass, Thermo Scientific) using Fluoromount-G (Southern Biotech) and allowed to dry overnight before imaging. All sections were imaged on a Keyence Biorevo BZ-9000 digital widefield microscope using a 40x/0.95 objective lens.

Supplemental tables & figures

Table S1. Summary of electrophysiological outcomes for individual organotypic brain slice cultures. Brain slice culture ID indicates the animal that culture was generated from (first digit) and an assigned slice number (second digit, posterior to anterior) within that animal (n=14 control; n=15 domoic acid). Outcomes of neuron-level analyses are spike rate (Hz) and burst rate (bursts/min) (mean ± standard error (SE)). Percent bursting neurons indicates the proportion of electrophysiologically active neurons that participate in bursting activity; percent PI neurons indicates the proportion of active neurons putatively identified as fast-spiking inhibitory neurons.

Treatment	Culture ID	# Active hippocampal neurons	Mean spike rate (Hz) (±SE)	# bursting neurons	% bursting neurons
Control	2-2	117	1.273 (±0.219)	112	95.7
Control	2-4	119	0.829 (±0.146)	117	98.3
Control	2-6	86	0.752 (±0.171)	82	95.3
Control	3-1	104	1.459 (±0.311)	100	96.2
Control	3-3	98	1.264 (±0.368)	92	93.9
Control	4-2	197	1.436 (±0.165)	189	95.9
Control	4-4	214	1.457 (±0.208)	198	92.5
Control	5-1	23	1.119 (±0.288)	14	60.9
Control	5-3	45	0.849 (±0.160)	43	95.6
Control	5-5	85	1.254 (±0.250)	58	68.2
Control	6-2	264	0.753 (±0.081)	254	96.2
Control	6-4	98	0.787 (±0.189)	96	98.0
Control	6-6	193	1.089 (±0.155)	187	96.9
Control	7-3	169	0.880 (±0.101)	168	99.4
DomA	2-1	63	0.805 (±0.163)	62	98.4
DomA	2-3	53	1.209 (±0.264)	52	98.1
DomA	3-2	126	0.973 (±0.137)	126	100
DomA	3-4	44	1.098 (±0.245)	43	97.7
DomA	3-6	138	0.377 (±0.047)	135	97.8

DomA	4-1	206	0.611 (±0.111)	203	98.5
DomA	4-3	89	0.681 (±0.117)	85	95.5
DomA	4-5	108	0.622 (±0.103)	106	98.1
DomA	5-2	29	1.194 (±0.386)	24	82.8
DomA	5-4	22	0.367 (±0.137)	20	90.9
DomA	6-1	143	0.576 (±0.094)	140	97.9
DomA	6-3	133	0.776 (±0.145)	133	100
DomA	7-2	82	0.538 (±0.089)	82	100
DomA	7-4	92	1.374 (±0.227)	90	97.8
DomA	7-6	131	1.465 (±0.198)	128	97.7

Table S1 (cont'd.)

Treatment	Culture ID	Average burst rate (bursts/min) (±SE)	# of PI neurons	% PI neurons
Control	2-2	1.202 (±0.174)	10	8.55%
Control	2-4	1.112 (±0.175)	2	1.68%
Control	2-6	1.226 (±0.202)	2	2.33%
Control	3-1	1.405 (±0.197)	8	7.69%
Control	3-3	1.387 (±0.243)	5	5.10%
Control	4-2	1.683 (±0.133)	9	4.57%
Control	4-4	1.085 (±0.106)	16	7.48%
Control	5-1	0.226 (±0.095)	1	4.35%
Control	5-3	1.799 (±0.355)	0	0.00%
Control	5-5	0.899 (±0.219)	5	5.88%
Control	6-2	0.905 (±0.078)	7	2.65%
Control	6-4	0.765 (±0.120)	4	4.08%
Control	6-6	1.353 (±0.153)	6	3.11%
Control	7-3	1.185 (±0.117)	3	1.78%

DomA	2-1	2.252 (±0.393)	1	1.59%
DomA	2-3	2.234 (±0.295)	1	1.89%
DomA	3-2	1.710 (±0.146)	0	0.00%
DomA	3-4	3.986 (±0.935)	1	2.27%
DomA	3-6	0.861 (±0.147)	0	0.00%
DomA	4-1	1.222 (±0.080)	3	1.46%
DomA	4-3	1.033 (±0.131)	0	0.00%
DomA	4-5	1.041 (±0.184)	1	0.93%
DomA	5-2	2.252 (±0.495)	0	0.00%
DomA	5-4	0.397 (±0.079)	0	0.00%
DomA	6-1	1.071 (±0.102)	0	0.00%
DomA	6-3	1.554 (±0.185)	0	0.00%
DomA	7-2	1.575 (±0.159)	1	1.22%
DomA	7-4	1.943 (±0.182)	2	2.17%
DomA	7-6	2.064 (±0.178)	2	1.53%

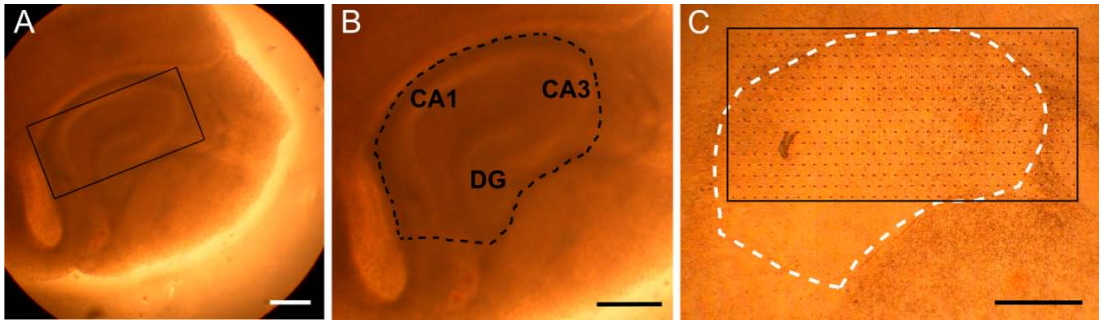


Figure S1. Photomicrographs of representative brain slice culture #6-3. (A) Organotypic brain slice culture on day 1 *in vitro* (DIV1); the 512-electrode array area is marked by the black rectangle. (B) Close-up of the hippocampal region (dashed line) in panel (A) with subregions (CA1, CA3, DG) identified. (C) The same tissue, post-recording (DIV17), showing the hippocampal region outlined in white (dashed line), and the electrode array outlined in black; the small black dots are individual electrodes. Scale bar = 500 µm.

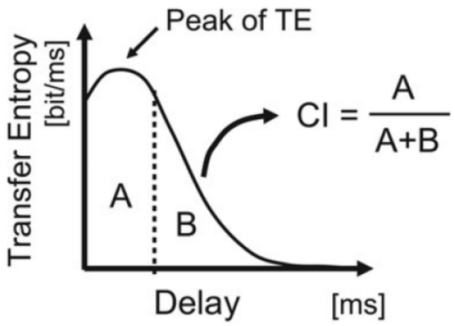
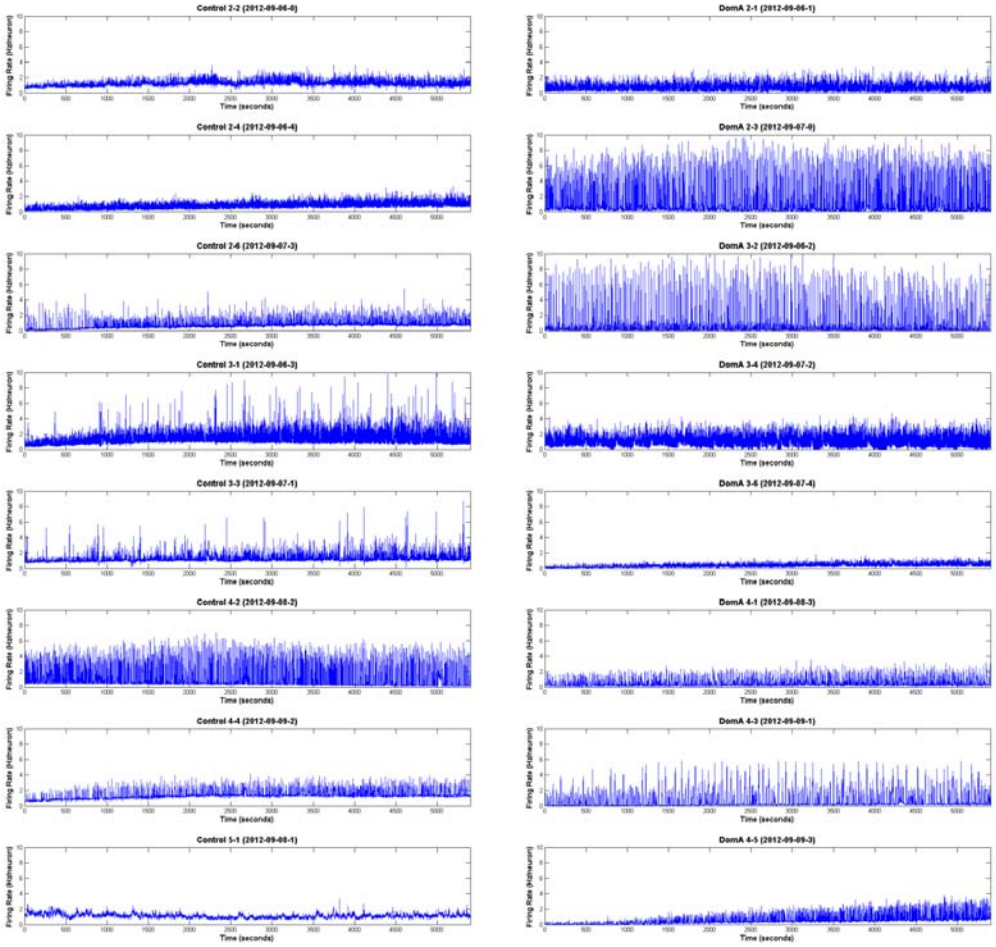


Figure S2. Determining coincidence index (CE). Transfer entropy is plotted as a function of delay (*d* in equation 3, above), and CI (equation 4, above) is defined as the ratio of the area under the peak (A) to the total area (A+B); from Shimono & Beggs (2014).



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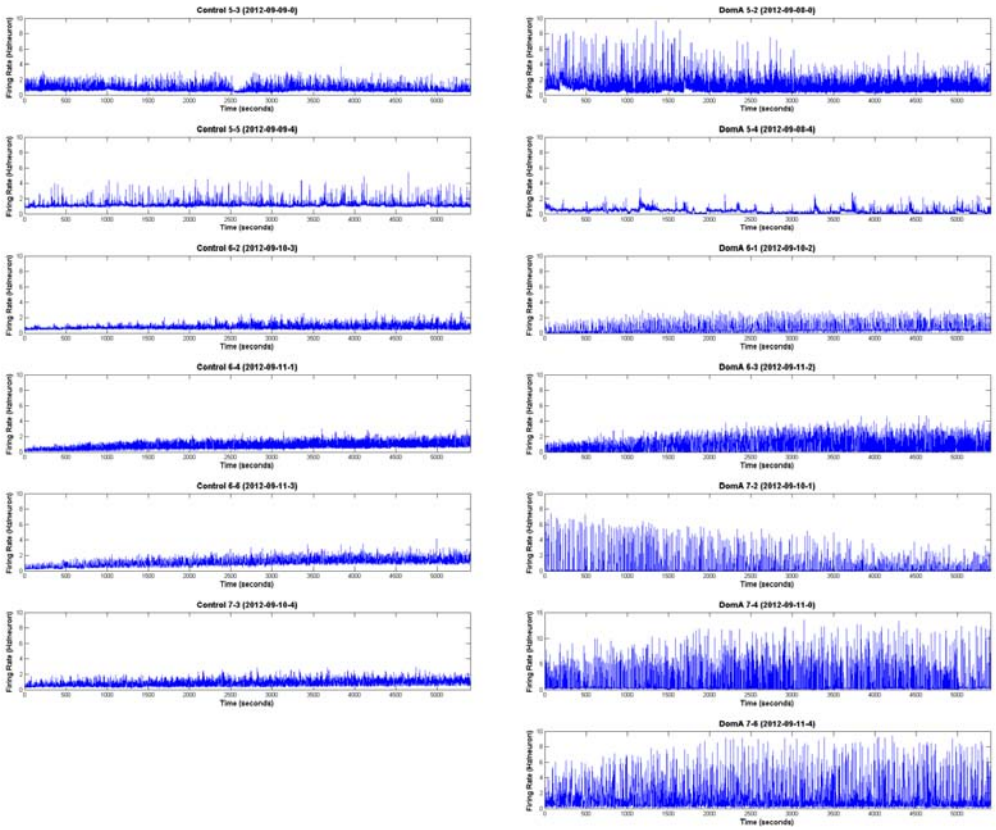
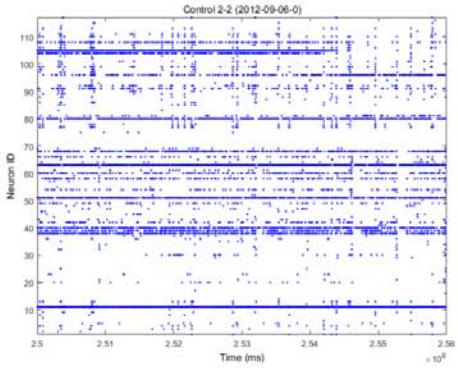
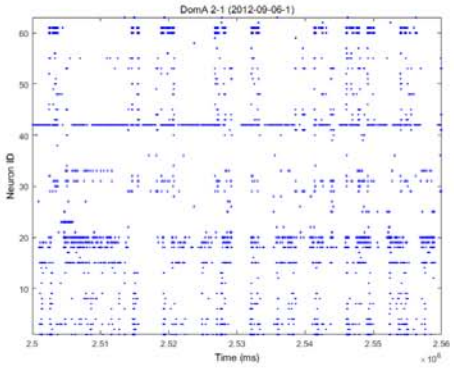
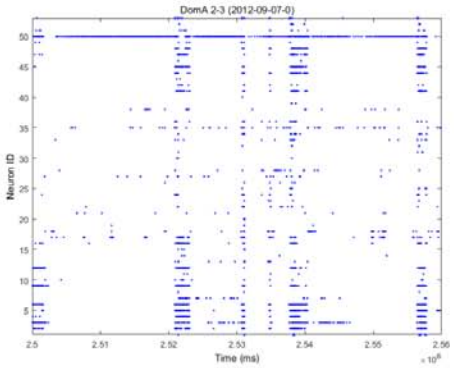
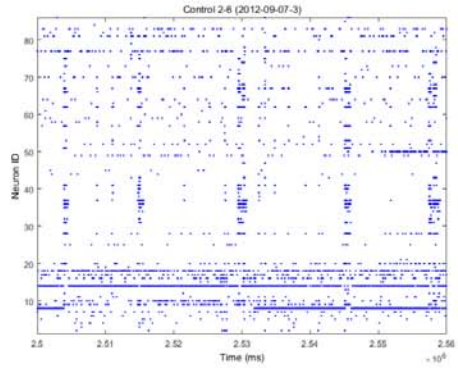


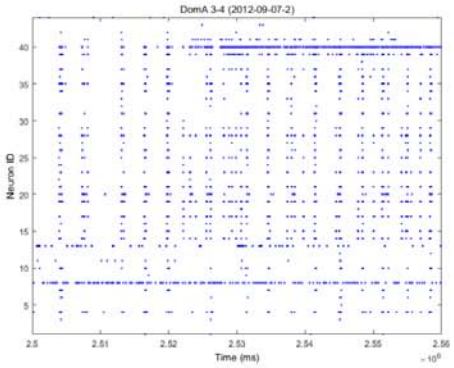
Figure S3. Activity profiles for all brain slice cultures over the 90 min (5,400 sec) recording period. Control brain slice recordings (n=14) are shown in the left column, and domoic acid-exposed cultures (n=15) are in the right column. Y-axis is the mean spike rate (Hz/neuron) of all electrophysiologically active neurons.

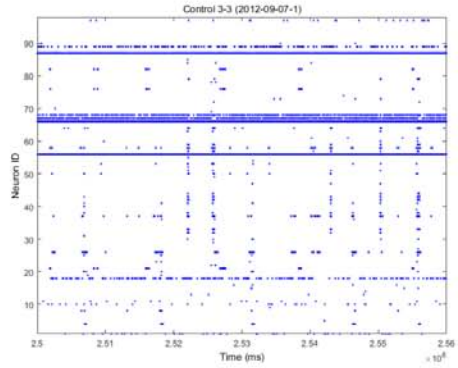


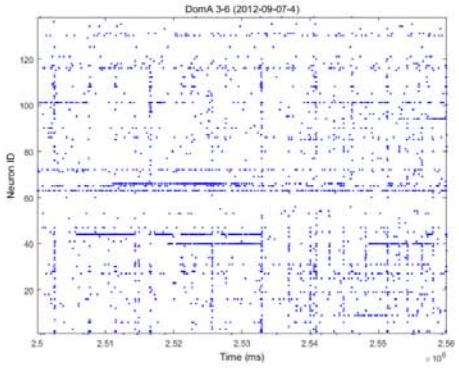












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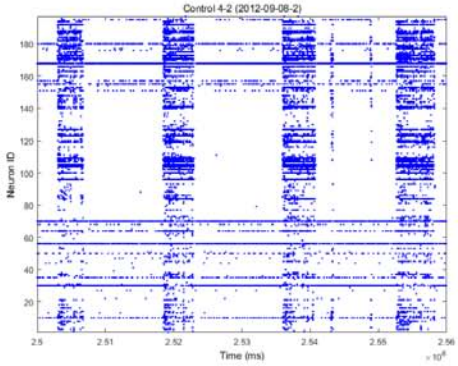

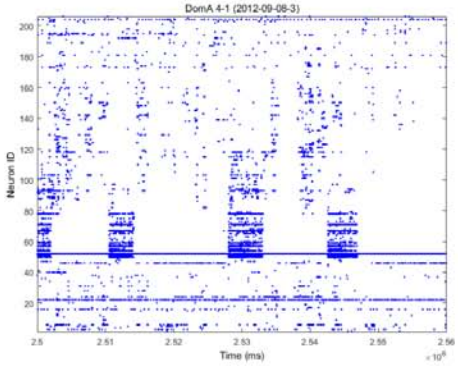

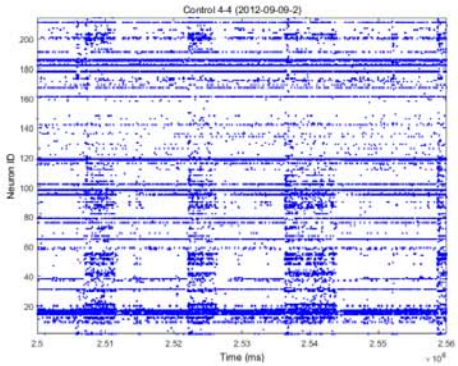

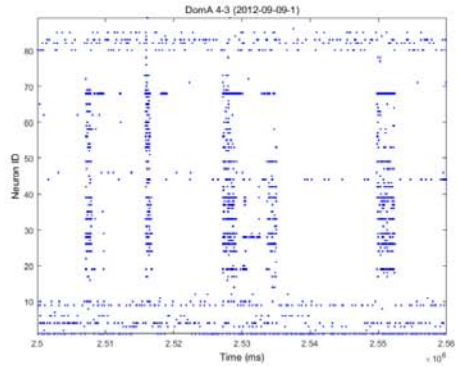

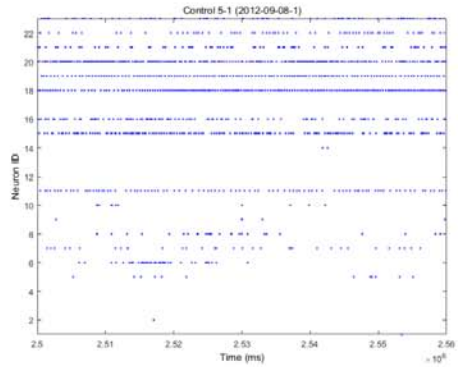

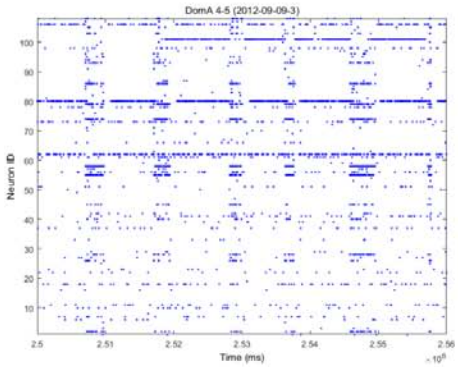

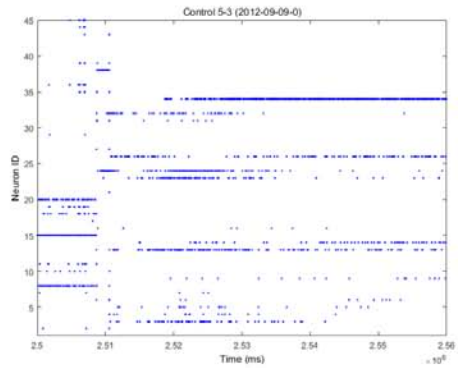

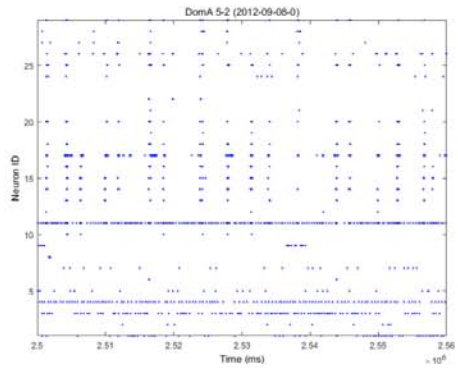

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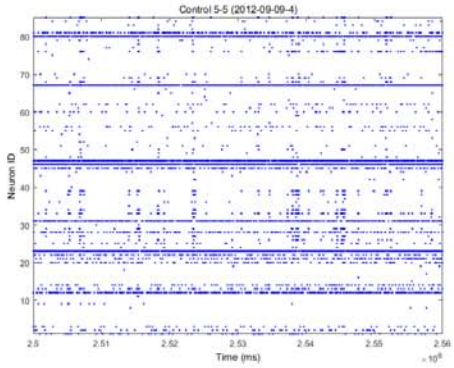

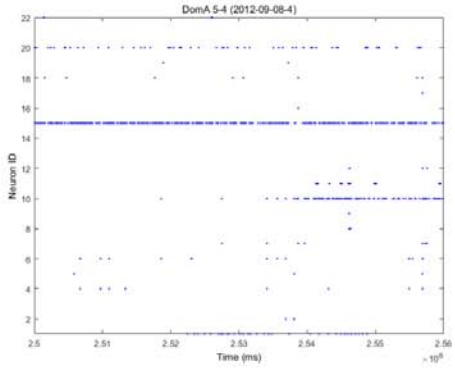

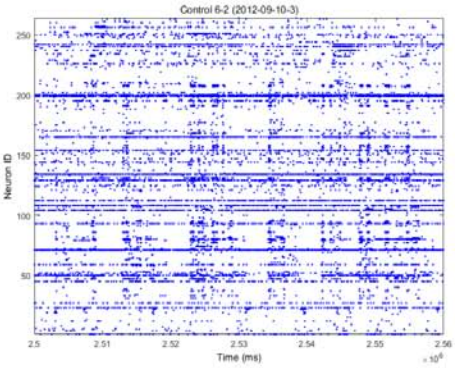

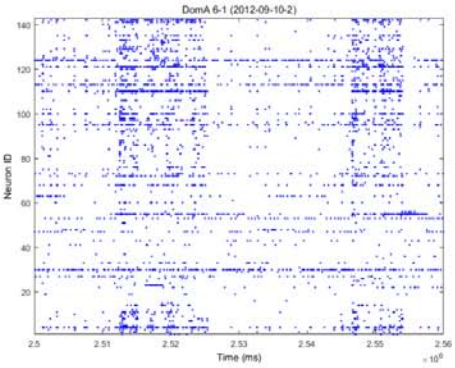

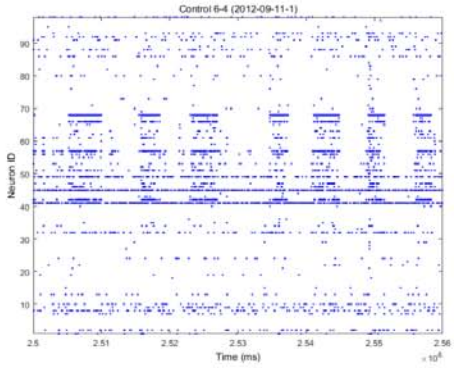

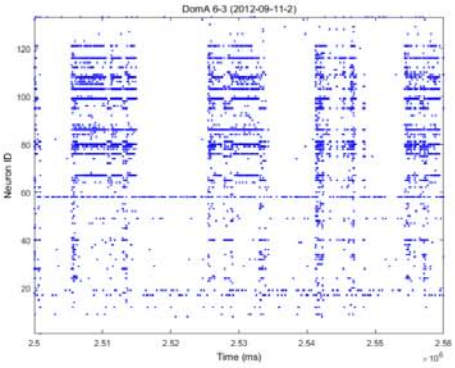

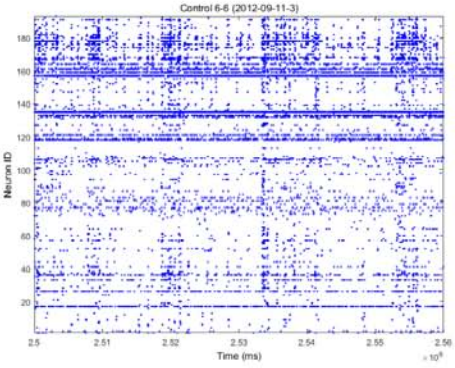

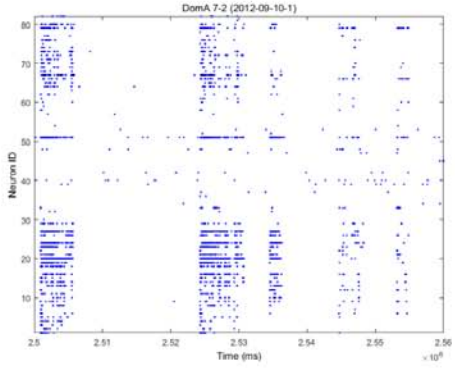

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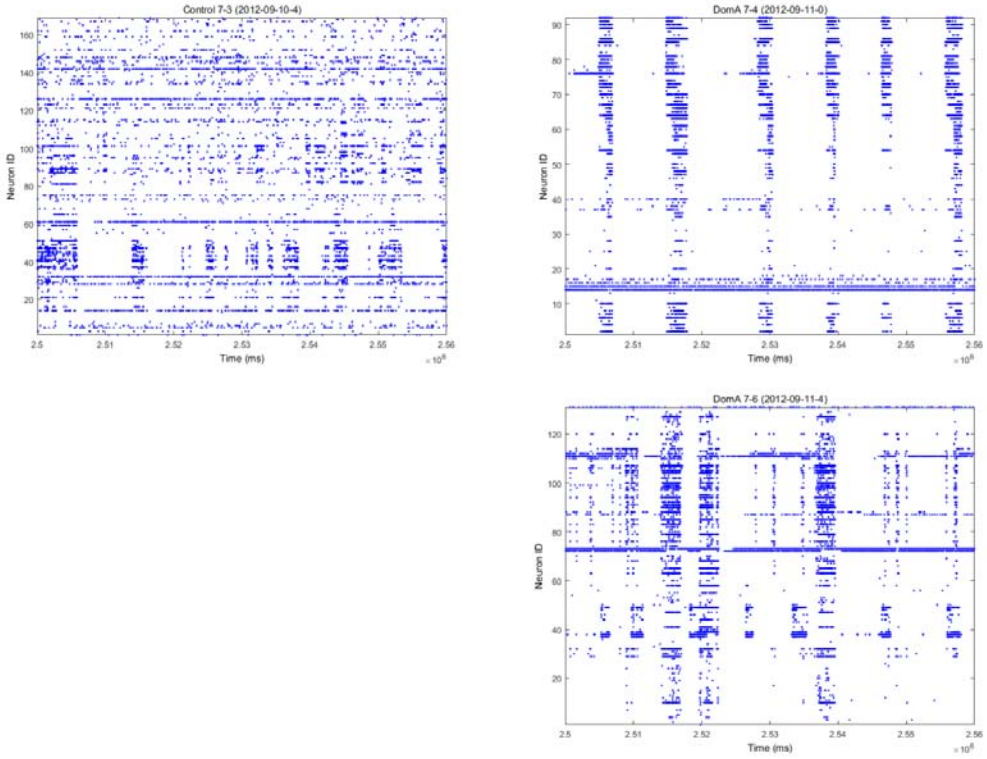

Figure S4. Raster plots for a representative one-minute recording interval (2500-2560 sec) for each brain slice culture. Control brain slice recordings are shown in the left column (n=14), and domoic acid-exposed cultures are in the right (n=15). Y-axis indicates the ID number for individual electrophysiological active neurons identified on the electrode array. Individual dots (•) indicate an action potential (spike) for that neuron.

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CHAPTER 4 – CHRONIC ASYMPTOMATIC DOMOIC ACID EXPOSURE DOES NOT CAUSE MORPHOLOGICAL CHANGES IN THE ADULT BRAIN

Emma M Hiolski, Preston S Kendrick, David J Marcinek, Kathi A Lefebvre, Donald R Smith

Abstract

Domoic acid, an algal-derived seafood toxin, exerts neurotoxic effects by over-stimulating neurons in the central nervous system. These effects have been well-studied in a variety of exposure paradigms, including acute and high-dose exposures (e.g., laboratory and dose-response studies), chronic variable-dose exposures (similar to what marine wildlife ingest through prey), and low-dose/asymptomatic exposures early in life (persistent effects of developmental exposure). However, there is a gap in knowledge about whether chronic low-level (i.e., asymptomatic) domoic acid exposure has harmful neurological effects, like cell death or injury, in the adult brain. We probed whether domoic acid-induced histomorphological changes could explain transient learning and memory impairments in adult mice following chronic asymptomatic domoic acid exposure. To quantify domoic acid-induced cell or neuron death, we immunostained mouse brain sections for DAPI and NeuN (neuronal nuclei) to achieve total cell and neuron counts, respectively. To assess whether chronic asymptomatic domoic acid exposure triggered a neuroinflammatory response, we measured GFAP (glial fibrillary acidic protein; astrocyte and neuroinflammation marker) by quantitative immunofluorescence. We also measured the effect of domoic acid exposure on a

subset of inhibitory neurons, parvalbumin-positive neurons, shown to be sensitive to neurological perturbation. We found no changes in histomorphological outcomes (hippocampal area, cell count, neuron count) in the hippocampus, no evidence of neuroinflammation (astrocyte activation), and no evidence of effects on number of parvalbumin-positive neurons or parvalbumin staining intensity. These results indicate that the transient behavioral deficits caused by chronic asymptomatic domoic acid exposure occurred in the absence of histopathology or neuroinflammation.

Introduction

Domoic acid is an excitatory neurotoxin produced by marine algae, and becomes concentrated in seafood during harmful algal blooms. Exposure has been documented to cause a variety of functional and histopathological effects in the vertebrate central nervous system, including: seizures, neuron death, neuroinflammation, brain lesions, and memory loss following acute/high-dose exposures (Ananth et al., 2001; Perl et al., 1990; Scallet et al., 2005, 1993); persistent changes in learning, memory, behavior, and hippocampal histomorphology following low-dose developmental exposures (Adams et al., 2009, 2008; Bernard et al., 2007; Doucette et al., 2004; Gill et al., 2012, 2010; Marriott et al., 2012; Ryan et al., 2011); and hippocampal atrophy and permanent learning and memory impairments in field-exposed marine wildlife following (i.e., chronic episodic exposure at extremely variable doses) (Cook et al., 2015; Goldstein et al., 2008). These studies create an important foundation for understanding the neurotoxicity and mode

of action for this toxin, and provide compelling evidence of the devastating impacts domoic acid has on marine mammals and sea birds.

However, chronic low-dose (i.e., causing no visible symptoms of excitotoxicity) exposure to domoic acid is relatively understudied. It is becoming more critical to address the potential health impacts of this exposure scenario because it is especially relevant for humans. We are well-protected from high-dose exposure to domoic acid by shellfish and seafood regulatory limits and monitoring efforts, but domoic acid is increasingly prevalent in the marine environment (Anderson et al., 2012; Hallegraeff, 1993; Lefebvre et al., 2016; Moore et al., 2008), highlighting the need to evaluate whether domoic acid exposure can produce neurological or histological effects in the adult brain, even in the absence of visible symptoms.

Although few studies employ a chronic asymptomatic domoic acid exposure paradigm, there is still evidence of neurological perturbation in the absence of overt excitotoxicity. Adult zebrafish chronically exposed to an asymptomatic dose of domoic acid (0.31 ± 0.03 µg domoic acid/g fish) become more neurologically sensitive to subsequent higher-dose exposures (Lefebvre et al., 2012a), respond with highly variable transcriptome changes in the brain characterized by activation of genes related to neurological function and development, and exhibit impaired mitochondrial function in the brain (Hiolski et al., 2014). Our collaborators have recently reported that chronic asymptomatic domoic acid exposure in adult mice (0.8 µg domoic acid/g mouse, 1x/week, 25 weeks) yields significant yet transient deficiencies in learning and memory in the absence of overt symptoms or excitotoxicity (unpublished data).

Using the same exposure paradigm, our objective was to determine if chronic asymptomatic domoic acid exposure produced histopathological consequences in the brains of adult mice that might be reflective of the transient behavioral deficits. We hypothesized that there might be changes in hippocampal area and cell or neuron death, and that domoic acid exposure would trigger mild/moderate neuroinflammation. Our secondary objective was to begin probing subtler neurological changes that could provide a basis for the observed learning and memory deficits. We hypothesized that fast-spiking, parvalbumin-positive inhibitory interneurons could be a target of low-dose domoic acid toxicity, initially based on our observations of electrophyisiological activity in organotypic brain slice cultures (Chapter 3).

In that study, we observed that the electrophysiological activity of fast-spiking neurons was suppressed in brain slice cultures exposed to low-dose domoic acid, and putatively identified these neurons as parvalbumin-positive inhibitory interneurons (Chapter 3). Evidence in the literature also indicates that parvalbumin-positive neurons are sensitive to seizures and other brain injuries, resulting in a reduction of parvalbumin-positive neuron numbers in the hippocampus (Best et al., 1993; Gorter et al., 2001; Johansen et al., 1990; Sloviter, 1991). Selective reductions in parvalbumin-positive neuron number has also been reported in adult (PND90) rat hippocampus following low-dose developmental domoic acid exposure (20 µg/kg, 1x subcutaneous injection daily over PND8-14) (Gill et al., 2010). Because parvalbumin-positive neurons play critical roles in balancing excitatory activity and maintaining network oscillations in the hippocampus (Hu et al., 2014) – these functions are critical for cognitive processes like learning and memory (Bartos et al., 2007), it is

feasible that the observed domoic acid-induced learning and memory dysfunction in mice could stem from disruption to this sub-population of neurons.

Methods

Domoic acid exposure

Female adult (~3 months old) wildtype C57BL/6NJ (Black 6) mice (Jackson Labs) were injected intraperitoneally once per week with either a dose of 0.8 µg domoic acid/g mouse (n=10) or vehicle (phosphate-buffered saline) only (n=10) for 22 weeks. Behavioral observations were conducted after each injection to confirm that no seizures or convulsive activity occurred.

Brain collection and tissue processing

After 22 weeks' exposure, mice were deeply sedated with 2.5% Avertin and trans-cardially perfused with 1x PBS, followed by 4% PFA (in PBS) for fixation. Brains were extracted, post-fixed in 4% PFA for 3-4 hours, and transferred to 30% sucrose for cryoprotection. Coronal sections were generated at 40 µm thickness with a Leica CM3050 S cryostat (Leica Biosystems), and stored at 4°C in 1x TBS (0.05% azide, preservative).

Immunostaining

Two hippocampal sections (200 µm apart) per animal, representing as similar a location within the hippocampus as possible for each animal, were selected and pseudo-randomly assigned a letter ID to blind the experimenter to treatment. All sections were processed free-floating for the following antigens: NeuN (neuronal

nuclei), GFAP (glial fibrillary acidic protein; astrocyte marker), and PV (parvalbumin; inhibitory neuron marker).

Primary antibodies used were: rabbit monoclonal anti-NeuN (neuronal nuclei (D3S3I) – Cell Signaling (#12943), 1:1000); rabbit polyclonal anti-GFAP (glial fibrillary acidic protein – Abcam (#ab7260), 1:1000); mouse monoclonal anti-PV (parvalbumin – Millipore (#MAB1572), 1:1000). Secondary antibodies used were: goat anti-rabbit IgG (Alexa Fluor647; Abcam (#ab150079), 1:1000); goat anti-rabbit IgG (Alexa Fluor488; Molecular Probes (#A11034), 1:1000); goat anti-mouse IgG (Alexa Fluor 555; Molecular Probes (#A21424), 1:1000).

All steps were performed on an orbital shaker table (Heidolph^{TM}). After being washed three times with tris-buffered saline (TBS), sections were blocked with 10% normal goat serum and permeabilized with 0.5% Triton-X for 2 hours at room temperature. Following another three washes with TBS, cultures were incubated with primary antibody overnight at 4°C (rabbit anti-GFAP) or for 48 hours at 4°C (rabbit anti-NeuN and mouse anti-PV).

Sections were then washed 3x with TBS and incubated with secondary antibody (goat anti-rabbit488 for GFAP, goat anti-rabbit647 for NeuN, goat anti-mouse555 for PV) for 2 hours at room temperature. Next, sections were washed 3x with TBS, DAPI stained for 10 min (Invitrogen D21490/DAPI-Fluoro- Pure Grade, 300 n*M* working solution), and washed 3x with TBS. Tissues were mounted on superfrost/Plus slides (Fisherbrand), then coverslipped (Slip-Rite #1.5 coverglass, Thermo Scientific) using Fluoromount-G (Southern Biotech) and allowed to dry overnight before imaging.

Imaging and image processing

All sections were imaged on a Keyence Biorevo BZ-9000 digital widefield microscope using a 40x/0.95 objective lens. For GFAP staining, 3D image stacks (1 µm z-spacing) were taken at two fields of view per hippocampal subregion (CA1, CA3, dentate gyrus (DG)) in both sections per animal; for NeuN and parvalbumin staining, image stacks were taken at three fields of view per subregion in anterior sections only (posterior sections were reserved for additional analyses, if needed). All imaging parameters (i.e., exposure time, detector gain and offset) were kept constant across all sections for each antigen. To generate images with which to hand-count parvalbumin-positive neurons and measure hippocampal area, both hippocampal hemispheres were imaged at 20x; a series of coarsely z-spaced (2 µm) stacks were tiled together to generate a merged and full-focused 'map' of each entire hippocampal hemisphere. All z-stacks were deconvolved using AutoQuant software (Media Cybernetics) for 10 iterations with default settings.

Cell counts and staining quantification

Imaris software was used to create 'surfaces' (3D digital renderings of fluorescent staining) for each outcome imaged at 40x. The total number of surfaces within each field of view yielded total cell count (DAPI staining), neuron count (NeuN staining), and parvalbumin-positive neuron count (PV staining). Total (summed) pixel fluorescence intensity values and volume measurements were also used to characterize $GFAP^+$ and PV^+ staining. Values for each field of view within an animal (4 FOVs for DAPI & GFAP; 3 FOVs for NeuN & PV) were summed to obtain a grand total for each outcome within each animal. With this approach, there is no need to

statistically account for within-animal variability, as there is only a single value per animal for each outcome.

Hippocampal 'map' images (20x tiled/merged images) were used to measure hippocampal areas (mm^2) by hand-tracing in ImageJ (version 1.50g, NIH, USA). Total hippocampal area and subregion-specific areas (CA1, CA3, DG) were measured for both hemispheres within a single section. An additional parvalbumin-positive neuron count was also done using ImageJ's Cell Counter feature to generate spatial density measurements.

Statistical analyses

All data were analyzed with JMP software (version 12.0.1, SAS Institute, Inc.), and were confirmed to have equal variance and normal distribution before analyses. Differences in cell counts, neuron counts, and GFAP staining between control and domoic acid groups were assessed by t test and are plotted as means ± standard error. Parvalbumin intensity outcomes (fluorescence intensity, volume (μm^3), and intensity/volume) were assessed on a per-neuron basis, accounting for the animal each neuron originated in with a mixed model ANOVA (treatment = fixed effect, animal = random effect), and are plotted as least squares mean ± least squares standard error from the model.

Results

Chronic domoic acid exposure did not cause visible damage in the hippocampus or cause quantifiable cell or neuron death

To determine whether chronic low-dose domoic acid exposure in adulthood caused gross histomorphological or cytotoxic effects, we measured hippocampal area and performed cell and neuron counts. Domoic acid exposure did not have a measurable effect on hippocampal area in brain sections of exposed mice compared to controls (Fig 1A) (mean area (mm^2) ± standard error for hippocampus: control 3.78 ± 0.11, domoic acid 3.78 ± 0.24, $p = 0.99$; CA1: control 1.25 ± 0.04, domoic acid 1.20 ± 0.09, $p = 0.61$; CA3: control 1.07 ± 0.06, domoic acid 1.14 ± 0.08, $p = 0.53$; DG: control 0.96 ± 0.2, domoic acid 0.97 ± 0.06, $p = 0.83$). There was also no significant effect on subregion-specific areas within the hippocampus (Fig 1B) (mean % of total hippocampal area for CA1: control 33.0 ± 0.68, domoic acid 31.6 ± 1.19, $p = 0.33$; CA3: control 28.4 ± 1.31, domoic acid 30.1 ± 1.09, $p = 0.32$; DG: control 25.4 ± 0.31, domoic acid 25.8 ± 0.47, $p = 0.49$). Domoic acid exposure did not result in overt cytotoxicity, as there was no quantifiable reduction in total cell numbers within any hippocampal subregion (Fig 2) (mean DAPI count ± standard error for CA1: control 2170 ± 64.5, domoic acid 2205 ± 30.5, $p = 0.64$; CA3: control 2224 ±91.1, domoic acid 2256 ± 73.8, $p = 0.79$; DG: control 4157 ± 94.2, domoic acid 4045 ± 148, $p = 0.54$). There was also no measurable effect on the number of neurons in any hippocampal subregion in the brains of exposed mice compared to controls (Fig 3) (mean total neuron count ± standard error for CA1: control 408 ± 10.2, domoic acid 375 ± 19.0, $p = 0.16$; CA3: control 504 ± 15.9, domoic acid 526 ± 37.8, $p = 0.61$; DG: control 733 ± 23.3, domoic acid 736 ± 53.0, $p = 0.96$).

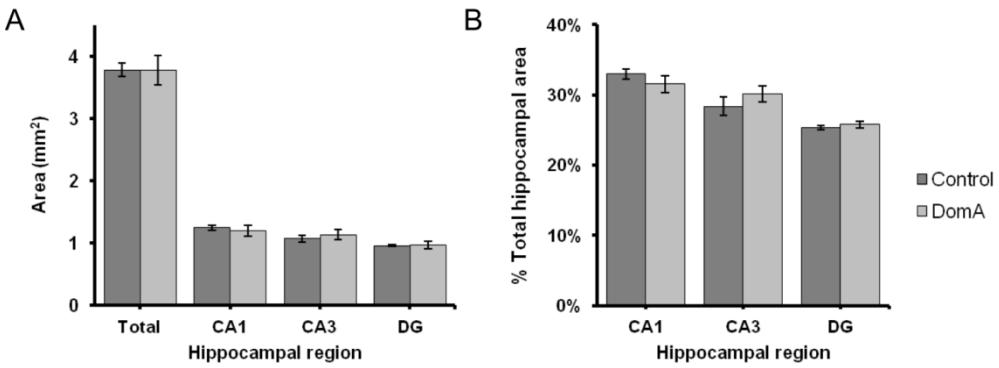

Figure 1. Hippocampal area was not affected by domoic acid treatment. (A) Total (i.e., both hemispheres summed within one tissue section) and subregion-specific hippocampal areas (mm^2) (mean ± SE) were combined across both hemispheres per animal (n=6 control, n=7 domoic acid). Significance was assessed by t test; $p > 0.05$ for all. (B) Subregion-specific proportions of total hippocampal area (mean ± SE) were also unaffected by treatment. Significance was assessed by t test; $p > 0.05$ for all.

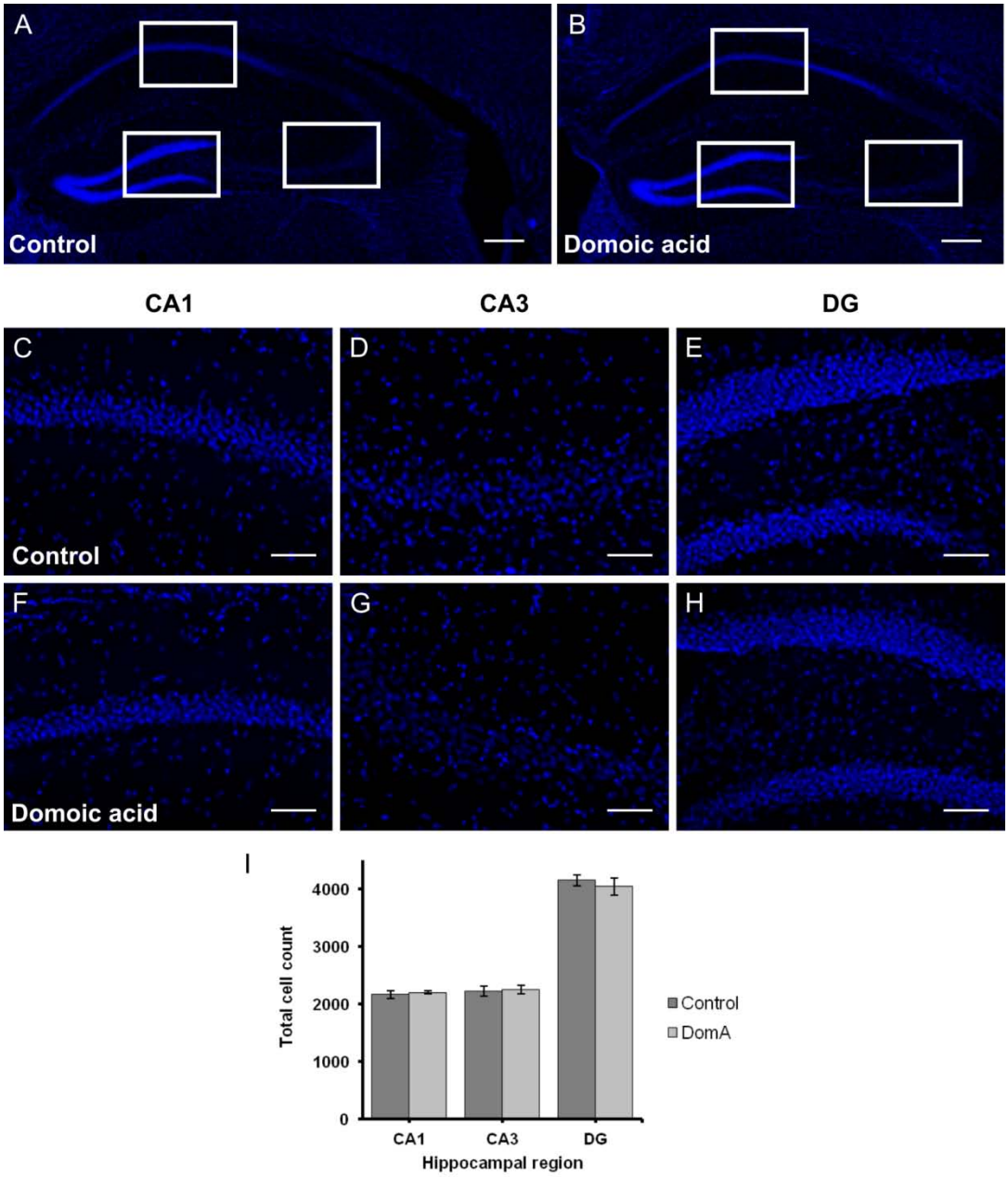

Figure 2. Domoic acid treatment did not affect total cell number in the hippocampus. Total cell counts (mean ± SE) were summed across four fields of view (sample fields of view are boxed in panels A and B) for each animal (n=6 control, n=7 domoic acid). Representative 20x merges of DAPI staining are shown for control (A) and domoic acid-exposed (B) brain sections (scale bar = 200 µm). Representative 40x images of DAPI staining (maximum projections of deconvolved z-stacks) are shown for control (C-E) and domoic acid-exposed (F-H) hippocampal subregions (CA1, CA3, DG) (scale bar = 50 µm). Quantification

of total cell counts is shown in (I). Significance was assessed by t test; $p > 0.05$ for all.

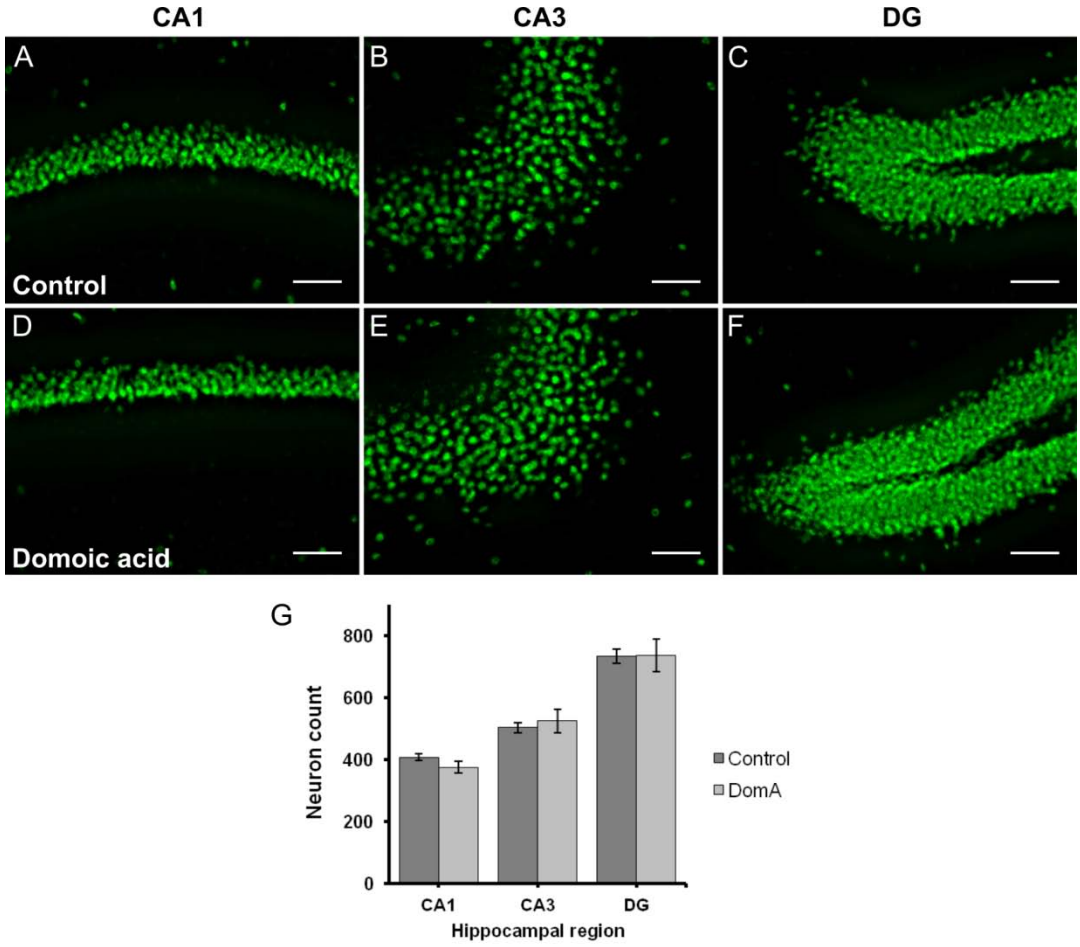

Figure 3. Neuron number was not reduced by domoic acid treatment. Total neuron counts (mean ± SE) were summed across three fields of view for each animal (n=6 control, n=7 domoic acid). Representative 40x images of NeuN staining (maximum projections of deconvolved z-stacks) are shown for control (A-C) and domoic acid-exposed (D-F) hippocampal subregions (CA1, CA3, DG) (scale bar = 50 µm). Quantification of total neuron counts is shown in (G). Significance was assessed by t test; $p > 0.05$ for all.

Chronic domoic acid exposure did not trigger a neuroinflammatory response

To quantify any neuroinflammatory response caused by chronic asymptomatic domoic acid exposure, we quantified $GFAP^+$ immunofluorescence as

an indicator of astrocyte activation in the hippocampi of mouse brain sections. $GFAP^+$ staining was quantified both by total pixel intensity, which corresponds to the amount of protein present, and by total volume (μm^3), which corresponds to astrocyte size, and would be increased if astrocytes became activated and exhibited hypertrophy. Domoic acid exposure did not result in overt neuroinflammation in any hippocampal subregion (Fig 4). Total $GFAP^+$ fluorescence intensity was not affected by chronic domoic acid exposure (Fig 4A) (mean total pixel intensity ± standard error for CA1: control 1.68E8 ± 1.66E7, domoic acid 1.66E8 ± 2.77E7, $p = 0.93$; CA3: control 9.74E7 ± 9.19E6, domoic acid 1.09E8 ± 3.00E7, $p = 0.72$; DG: control 1.78E8 ± 1.70E7, domoic acid 2.21E8 ± 5.92E7, $p = 0.52$). We also observed no significant difference in astrocyte ($GFAP^+$) volume (μm^3) due to domoic acid exposure (Fig 4B) (mean total volume (μm^3) ± standard error for CA1: 199,000 ± 15,900, domoic acid 200,000 ± 28,900, $p = 0.96$; CA3: control 129,000 ± 11,900, domoic acid 145,000 ± 35,500, $p = 0.68$; DG: control 227,000 ± 18,100, domoic acid 257,000 ± 55,300, $p = 0.63$).

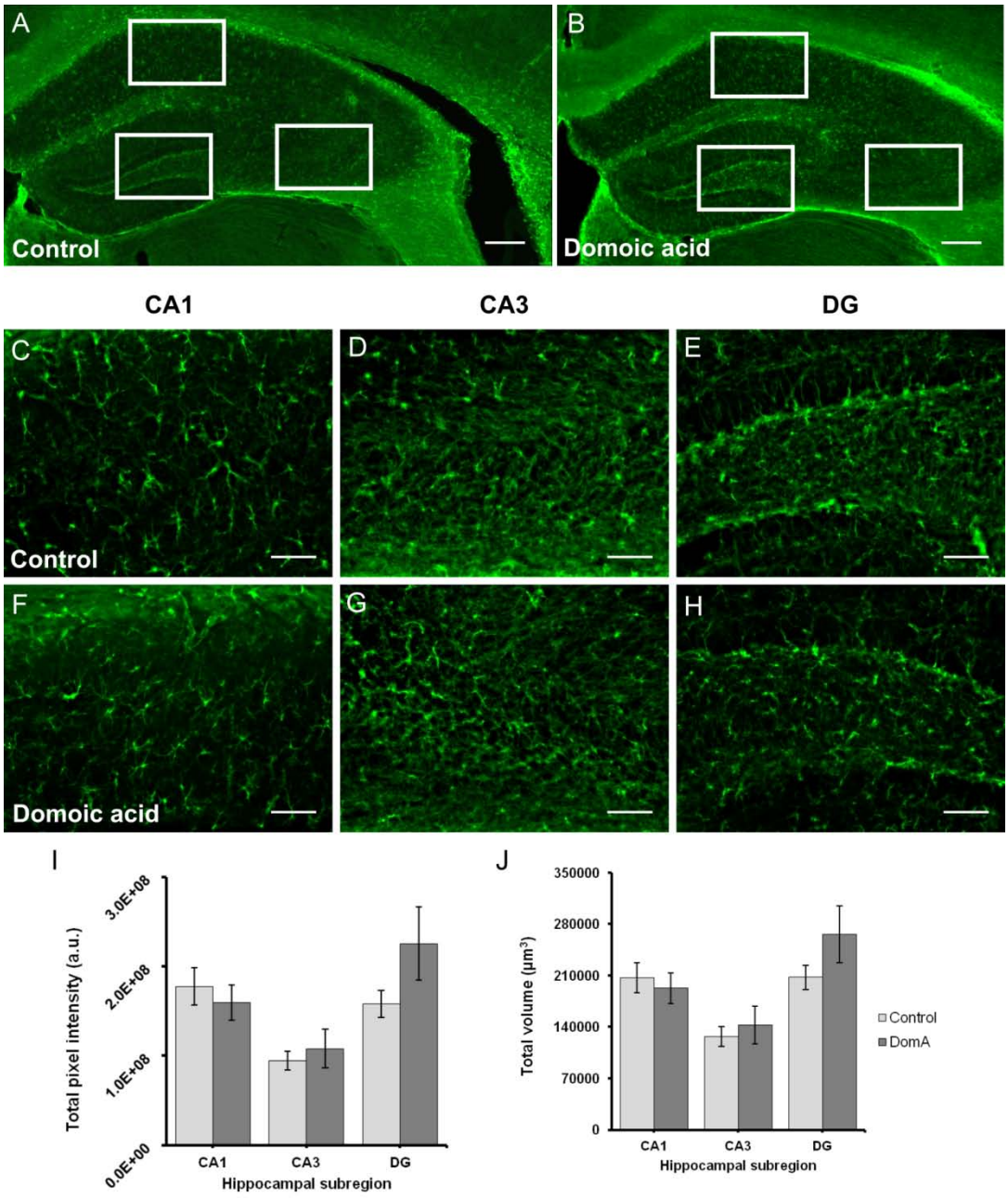

Figure 4. Domoic acid treatment did not significantly increase GFAP staining intensity or volume. Total pixel fluorescence intensity (arbitrary units (a.u.)) and volume (μm^3) for $GFAP^+$ staining was summed over four fields of view (sample fields of view are boxed in panels A and B) for each animal (mean ± SE) (n=6 control, n=7 domoic acid). Representative 20x merges of GFAP staining are shown for control (A) and domoic acid-exposed (B) brain sections (scale bar = 200 µm). Representative 40x images of GFAP staining (maximum projections of deconvolved z-stacks) are shown for control (C-E) and domoic acid-exposed (F-

H) hippocampal subregions (CA1, CA3, DG) (scale bar = 50 µm). Quantification of fluorescence intensity is shown in (I), and quantification of $GFAP^+$ volume (μm^3) is shown in (J). Significance was assessed by t test; $p > 0.05$ for all

Chronic domoic acid exposure did not affect parvalbumin-positive neuron number or alter parvalbumin staining intensity

To assess whether chronic domoic acid exposure perturbed parvalbumin-positive neurons – a subset of specialized inhibitory neurons that play critical roles in moderating behavior and balancing excitatory activity – we performed neuron counts, measured neuron density within hippocampal subregions, and quantified the fluorescence intensity of individual parvalbumin-positive neurons in the hippocampi of control and exposed mice. Prolonged exposure to low-dose domoic acid did not affect the number (Fig 5A) (mean parvalbumin-positive neuron count ± standard error for CA1: 20.7 ± 2.12, domoic acid 18.9 ± 1.94, $p = 0.54$; CA3: control 19.7 ± 2.47, domoic acid 22.3 ± 1.80, $p = 0.41$; DG: control 7.67 ± 1.05, domoic acid 8.43 ± 0.43, $p = 0.53$) or spatial density (Fig 5B) (parvalbumin-positive neuron count per mm^2 for total hippocampus: control 41.9 ± 1.96, domoic acid 43.7 ± 2.05, $p = 0.54$; CA1: control 51.4 ± 3.82, domoic acid 58.1 ± 8.42, $p = 0.49$; CA3: control 55.4 ± 5.10, domoic acid 55.8 ± 3.06, $p = 0.94$; DG: control 22.6 ± 1.77, domoic acid 20.4 ± 1.62, $p = 0.37$) of parvalbumin-positive inhibitory neurons in any hippocampal subregion.

The sum fluorescence intensity (a.u.) of parvalbumin-positive neurons was not significantly different in any hippocampal subregion (Fig. 6A) (least squares intensity (a.u.) mean ± least squares error in CA1: control 237,000 ± 29,300, domoic acid 259,000 ± 27,500, $p = 0.74$; CA3: control 212,000 ± 23,800, domoic acid

202,000 ± 21,600, p = 0.49; DG: control 366,000 ± 48,800, domoic acid 323,000 ± 43,600, p = 0.78). The volume (μm^3) of parvalbumin-positive neurons was also not significantly different between control and domoic acid-treated groups (Fig. 6B) (least squares mean intensity (μm^3) ± least squares error in CA1: control 1010 ± 39.3, domoic acid 987 ± 37.8, p = 0.60; CA3: control 1140 ± 64.0, domoic acid 1200 ± 57.3, p = 0.76; DG: control 1210 ± 111, domoic acid 1250 ± 100, p = 0.52. Parvalbumin-positive staining intensity normalized to neuron volume (intensity/μm^3) was also unaffected by domoic acid treatment (Fig 6C) (least squares mean parvalbumin-positive fluorescence intensity/μm^3 ± least squares error in CA1: control 237 ± 26.0, domoic acid 261 ± 24.4, p = 0.51; CA3: control 184 ± 15.6, domoic acid 171 ± 14.0, p = 0.54; DG: control 298 ± 28.7, domoic acid 265 ± 25.3, p = 0.41).

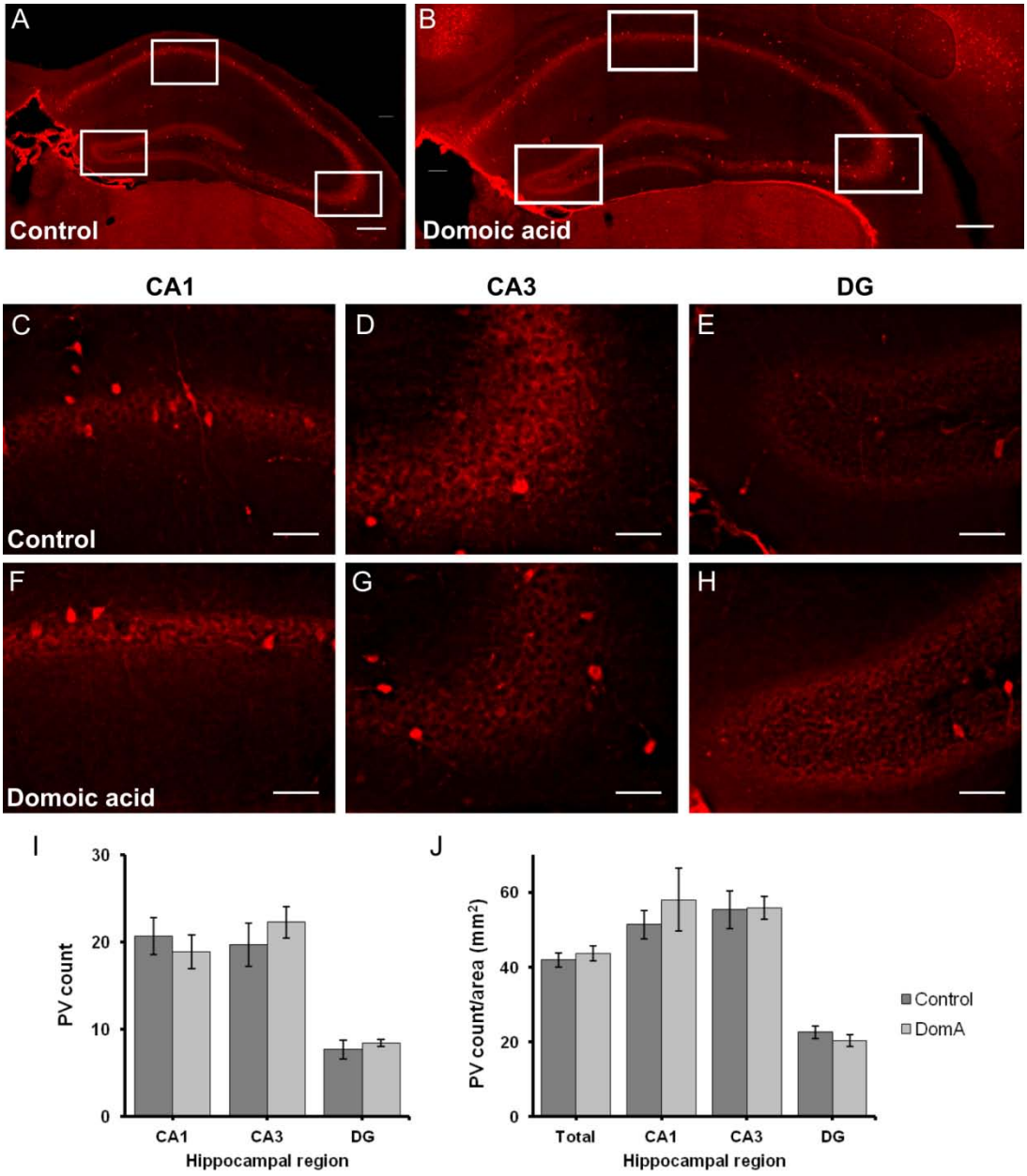

Figure 5. Neither parvalbumin-positive (PV) neuron number nor density within each subregion were affected by domoic acid treatment.
Representative 20x merges of parvalbumin staining are shown for control (A) and domoic acid-exposed (B) brain sections (scale bar = 200 µm). Representative 40x images of parvalbumin staining (maximum projections of deconvolved z-stacks) are shown for control (C-E) and domoic acid-exposed (F-H) hippocampal subregions (CA1, CA3, DG) (scale bar = 50 µm). (I) Total parvalbumin-positive (PV) neuron counts (mean ± SE) were summed across three fields of view within a subregion (see boxes in panels A and B for sample fields of view for each

subregion) for each brain section (n=6 control, n=7 domoic acid). Significance was assessed by t test; $p > 0.05$ for all. (J) Parvalbumin-positive neuron counts were normalized to hippocampal area (mm^2) (mean ± SE) to assess spatial density. Significance was assessed by t test; $p > 0.05$ for all.

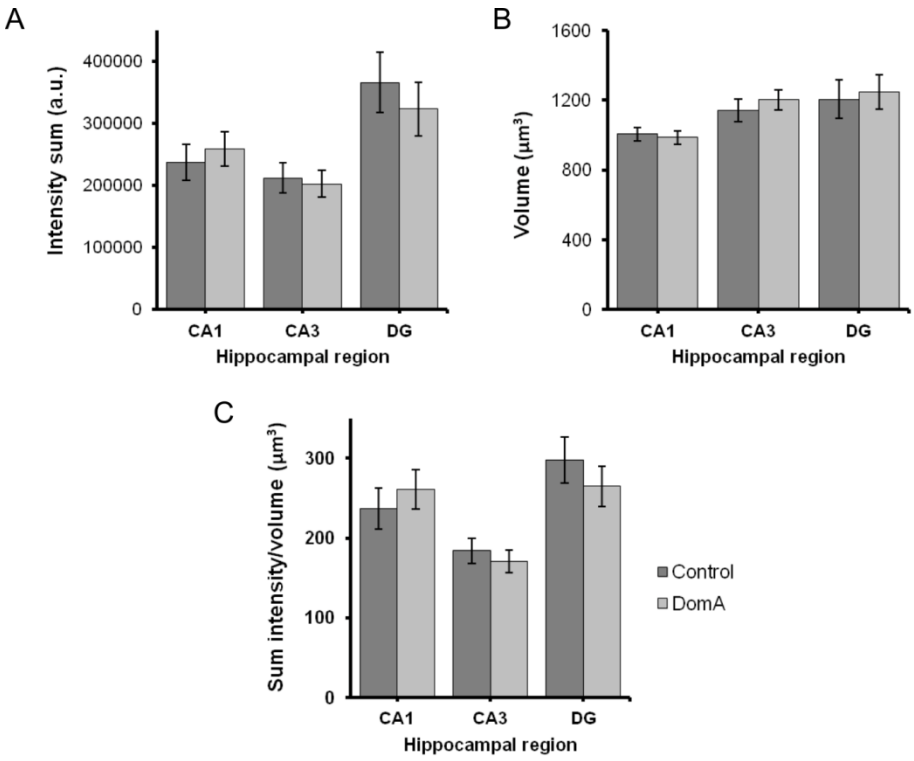

Figure 6. Domoic acid treatment did not affect parvalbumin staining intensity. (A) Total fluorescence intensity (least squares mean ± SE) averaged for all neurons identified by Imaris' algorithm (n=6 control animals with n=12-26, 10-29, and 4-11 parvalbumin-positive neurons in CA1, CA3, and DG, respectively; n=7 domoic acid animals with n=14-26, 16-31, and 7-10 PV-positive neurons in CA1, CA3, and DG, respectively). (B) Parvalbumin-positive neuron volume (μm^3) (least squares mean ± SE) for all neurons identified in control and domoic acid-exposed hippocampal subregions. (C) Fluorescence intensity was normalized to neuron volume (μm^3) (least squares mean ± SE) for all neurons. Significance was with a mixed model ANOVA (treatment = fixed effect, animal = random effect); $p > 0.05$ for all.

Discussion

Prolonged, low-level domoic acid exposure did not cause gross hippocampal damage or overt cell or neuron death

Our first goal was to measure any morphological changes in the hippocampus induced by chronic asymptomatic domoic acid exposure. We looked for signs of atrophy and cell/neuron death based on the well-documented neurotoxicity of acute symptomatic exposures. Domoic acid exposure has been shown to cause brain lesions and hippocampal atrophy in humans following consumption of contaminated shellfish (31-128 mg domoic acid/100 g mussel tissue) (Perl et al., 1990; Teitelbaum et al., 1990). Laboratory studies with single-dose exposures of 0.5-4 mg domoic acid/kg body weight (i.v. or i.p. injection) cause brain lesions and neuron death in non-human primates (Scallet et al., 1993) and rodents (Ananth et al., 2001; Scallet et al., 2005). Sea lions consume domoic acid at a range of doses, reaching up to mg/kg levels during harmful algal blooms (Bejarano et al., 2007), and repeated exposure has been linked to hippocampal atrophy and neuronal necrosis, leading to permanent deficits in spatial learning and memory (Montie et al., 2010; Silvagni et al., 2005). These histopathological changes are due to domoic acid-induced glutamatergic neuron death through apoptotic and necrotic pathways (Ananth et al., 2001; Carvalho Pinto-Silva et al., 2008; Erin and Billingsley, 2004).

We hypothesized that chronic asymptomatic domoic acid exposure might have caused histopathology in the hippocampus, based on the learning and memory deficits measured by our collaborators (unpublished data). The hippocampi of exposed animals, however, did not exhibit any atrophy (Fig 1), cell death (Fig 2), or neuron death (Fig 3), indicating that our exposure scenario did not induce gross

histopathology. This is consistent with histological assessment of zebrafish brains following 36 weeks of asymptomatic domoic acid exposure (0.31 ± 0.03 µg domoic acid/g fish, 1-2x/week) that showed no evidence of lesions or atrophy in any region (Hiolski et al., 2014).

A neuroinflammatory response was not triggered by chronic low-dose domoic acid exposure

In the absence of atrophy and cell/neuron death, we hypothesized that the transient learning and memory deficits seen in mice could have been triggered by a neuroinflammatory response to neuronal injury. Following high-dose exposures, domoic acid has been shown to initiate glial activation in response to neuron injury and death (Ananth et al., 2003, 2001; Appel et al., 1997; Kirkley et al., 2014; Mayer et al., 2001; Scallet et al., 1993). During a glial response to brain injury, microglia and astrocytes ($GFAP^+$) are activated and recruited to the injury site to assist with removal of cellular debris and to support neuron health (Hanisch and Kettenmann, 2007; Neumann et al., 2009). Astrocyte activation is characterized by hypertrophy, an enlargement of the cell body and processes (Pekny and Nilsson, 2005).

In our measurements of $GFAP^+$ staining, however, no astrocyte hypertrophy was present (Fig 4), indicating that a neuroinflammatory response was not induced by chronic asymptomatic exposure to domoic acid. This is consistent with gene expression data from the brains of zebrafish chronically exposed to low-dose domoic acid: Throughout 36 weeks of bi-weekly exposure, no upregulation of *gfap* genes was detected, indicating that no neuroinflammatory response was activated (Hiolski et al., 2014).

Domoic acid exposure did not reduce parvalbumin-positive inhibitory interneuron number or affect parvalbumin staining intensity

In the absence of histopathology and neuroinflammation, we hypothesized that the transient functional deficits in learning and memory caused by chronic low-level domoic acid exposure could be mediated through a subpopulation of neurons, specifically fast-spiking parvalbumin-positive inhibitory neurons. This hypothesis was based on domoic acid-induced suppression of electrophysiological activity in fast-spiking neurons in organotypic brain slice cultures (Chapter 3), evidence that parvalbumin-positive neurons are sensitive to neurological disruption and injury (Best et al., 1993; Gill et al., 2010; Gorter et al., 2001; Johansen et al., 1990; Sloviter, 1991), and the importance of their role in maintaining proper neuronal network activity and function (Bartos et al., 2007; Hu et al., 2014). We saw no indication that parvalbumin-positive neuron number or spatial density was affected by chronic low-dose domoic acid exposure (Fig 5), consistent with our finding above of no domoic acid-induced cellular or neuronal death.

To begin probing subtler effects of chronic low-dose domoic acid exposure, we examined the staining intensity and volume of parvalbumin-positive neurons in the hippocampus, as alterations in the density of parvalbumin-positive staining within neurons could potentially affect neuronal signaling ability (Hu et al., 2014). However, there were no measureable domoic acid-induced changes in staining intensity within parvalbumin-positive neurons (Fig 6). It is important to note that absence of a significant effect does not necessarily mean that this neuronal subpopulation's electrophysiological activity is not impacted; experiments beyond the scope of this

project would be required to determine whether brain activity and connectivity was affected in these chronically exposed mice.

Conclusion

Our chronic asymptomatic domoic acid exposure paradigm did not cause any hippocampal atrophy, cell/neuron death, or neuroinflammation. These results indicate that domoic acid exposure may have profound neurological effects – as evidenced by the significant learning and memory deficits measured by our collaborators – even in the absence of histopathology. Additionally, while the effect of domoic acid on parvalbumin-positive neurons was not measureable by immunohistochemistry, it does not preclude the possibility that these neurons could be impacted in other ways (e.g., electrophysiologically), or that domoic acid is affecting other targets.

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CHAPTER 5 – CONCLUSIONS

Domoic acid poisoning significantly impacts marine wildlife health, affecting hundreds of mammals and sea birds (Gulland, 2000; Scholin et al., 2000; Sierra-Beltran et al., 1997; Work et al., 1993) through contaminated prey items like baitfish and shellfish. The health risks that domoic acid may pose to humans, however, are comparatively unknown. Although we are well-protected from high-dose exposures through monitoring efforts and harvesting regulations enforced by public health and fish and wildlife departments, seafood from commercial and recreational harvests that contains domoic acid concentrations below the regulatory limit (20 µg domoic acid/g shellfish tissue) is still available for human consumption. The frequency of domoic acid contamination in seafood is likely to increase as the frequency and duration of harmful algal blooms rises (Anderson et al., 2012; Hallegraeff, 1993; Moore et al., 2008), and the range over which domoic acid is detected expands (Lefebvre et al., 2016). These changes make it critical to understand whether chronic exposure to low levels of domoic acid poses a significant health risk to humans and wildlife, as the neurological effects of frequent low-level (i.e., asymptomatic or below the regulatory limit) exposures over a long duration are unknown.

Dissertation summary

To address this knowledge gap, I used an interdisciplinary approach to uncover subtle changes in the brain caused by repeated low-dose (i.e., asymptomatic) domoic acid exposure. First, I used a genomic approach to discover changes in gene transcription and mitochondrial function in adult zebrafish brains

throughout the course of chronic, asymptomatic domoic acid exposure (Hiolski et al., 2014). I found that transcription of genes related to neurological function and development were significantly altered throughout the exposure duration, but that the transcriptome response was highly variable across the six sampling time points (2, 6, 12, 18, 24, and 36 weeks exposure). There was little to no overlap of genes at any of these time points, and no apparent similarities to the transcriptional profile of a transgenic mouse model of chronic moderate excess glutamate release (*glud1* transgene (Bao et al., 2009)). These results indicate that the transcriptional response to domoic acid exposure in the brain is dynamic, possibly indicating a constantly changing cellular response to compensate for repetitive perturbation, and distinct from congenital glutamate dysregulation. Additionally, I reported domoic acid-induced impairments in mitochondrial function, partially offset by compensatory increases in the mitochondrial content of brain cells, suggesting that repetitive low-level exposure does have fundamental cellular level consequences that could contribute to chronic neurological health consequences.

To learn more about how low-dose domoic acid could potentially impede brain function in exposed wildlife or humans, I worked with Alan Litke and Shinya Ito (Santa Cruz Institute for Particle Physics) to record electrical activity from hundreds of neurons in organotypic brain slice cultures. Organotypic brain slice cultures maintain cellular composition and organization of corticohippocampal tissue, and provide direct access to hippocampal circuits. We used a state-of-the-art 512-electrode array to quantify domoic acid's effects on the activity and connectivity of developing neural networks. I found a significant reduction in the number of electrophysiologically active neurons in domoic acid-exposed brain slice cultures,

with no measureable change in the number of cells or neurons (i.e., no cell/neuron death). This indicates that in the absence of overt cytotoxicity, domoic acid exposure still contributed to persistent electrophysiological effects, namely significant alterations in the spiking pattern of neurons from exposed brain slice cultures (Chapter 3). Without altering the overall mean spike rate (Hz), domoic acid exposure significantly increased the proportion of neurons that participated in bursting activity (temporally clustered spike trains) (Chapter 3). Additionally, the functional connectivity within neuronal networks of domoic acid-exposed cultures was significantly enhanced compared to controls, and a subset of fast-spiking neurons (putatively identified as parvalbumin-positive inhibitory neurons) was silenced in domoic acid-exposed cultures (Chapter 3). Taken together, these results provide evidence that low-dose (i.e., non-cytotoxic) domoic acid exposure can disrupt electrophysiological activity and interconnectedness in neural networks.

I also collaborated with Kathi Lefebvre and Dave Marcinek (NOAA Fisheries and University of Washington, Seattle) to determine how prolonged asymptomatic domoic acid exposure affects the adult brain. They reported significant impairments to learning and memory in adult mice following chronic asymptomatic domoic acid exposure. My specific objective was to uncover any histological changes that could link cellular-/neuron-level outcomes with the observed deficits in learning and memory. I used immunohistochemistry to: 1) assess whether our domoic acid exposure paradigm induced gross histopathology (hippocampal atrophy, cell and neuron death, e.g.) or initiated a neuroinflammatory response; and 2) measure the number of parvalbumin-positive neurons and parvalbumin staining intensity in the neurons of exposed mice compared to controls (Chapter 4). I found no significant

effect of domoic acid exposure on any parvalbumin staining outcomes, indicating that the neuron sub-population was not decimated, nor was the quantity of parvalbumin within individual neurons affected. Even in light of these negative results, it is still possible that domoic acid could exert electrophysiological effects on parvalbumin-positive neurons that are not measurable through immunostaining.

Broader impacts and future directions

Collectively, my dissertation research indicates that chronic exposure to asymptomatic domoic acid can significantly alter gene expression, cellular energetics, and neuronal network activity and connectivity, all in the absence of overt excitotoxicity and measurable neuron/cell death in the brain. These results have implications for the ability of repetitive asymptomatic domoic acid exposure to perturb neurological health. Chronic altered gene expression could change protein availability and disrupt neuronal homeostasis, and impaired mitochondrial function could impede neuronal ability to return to homeostasis as exposure duration increases. Over time, these changes could lead to impaired neuron function. Altered activity patterns and increased connectivity in the hippocampus could have lasting effects on the developing brain, resulting in impaired neurological function and altered behavior, especially given the observation of transient learning and memory impairments in adult mice chronically exposed to asymptomatic domoic acid. These repeated asymptomatic exposures are especially important to study in the context of human health because they are likely far more common than symptomatic exposures, as humans are more likely to be exposed only to levels of domoic acid below the regulatory limits imposed on the seafood industry.

Ultimately, these projects can provide foundational information to help answer long-term questions like: Is the regulatory limit protective enough? Should it be changed? What other monitoring and/or regulatory efforts may need to be included? When do the effects of chronic/low-dose domoic acid exposure become permanent? Are any effects reversible? How might the neurotoxic effects of domoic acid interact with those of other persistent marine pollutants (e.g., mercury, organochlorines, etc.)? Improving our understanding of the links between oceans and human health can help balance the need to protect livelihoods and cultural traditions with the need to protect people from potential health risks.

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