

# Repeated low level domoic acid exposure increases CA1 VGluT1 levels, but not bouton density, VGluT2 or VGAT levels in the hippocampus of adult mice

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## ABSTRACT

Domoic acid (DA) is a neurotoxin produced during harmful algal blooms that accumulates in marine organisms that serve as food resources for humans. While acute DA neurotoxicity can cause seizures and hippocampal lesions, less is known regarding how chronic, subacute DA exposure in adulthood impacts the hippocampus. With more frequent occurrences of harmful algal blooms, it is important to understand the potential impact of repeated, low-level DA exposure on human health. To model repeated, low-dose DA exposure, adult mice received a single low-dose ( $0.75 \pm 0.05 \mu\text{g/g}$ ) of DA or vehicle weekly for 22 consecutive weeks. Quantitative immunohistochemistry was performed to assess the effects of repeated, low-level DA exposure on hippocampal cells and synapses. Vesicular glutamate transporter 1 (VGluT1) immunoreactivity within excitatory boutons in CA1 of DA-exposed mice was increased. Levels of other vesicular transporter proteins (*i.e.*, VGluT2 and the vesicular GABA transporter (VGAT)) within boutons, and corresponding bouton densities, were not significantly altered in CA1, CA3, or dentate gyrus. There were no significant changes in neuron density or glial fibrillary acidic protein (GFAP) immunoreactivity following chronic, low-dose exposure. This suggests that repeated low doses of DA, unlike high doses of DA, do not cause neuronal loss or astrocyte activation in hippocampus in adult mice. Instead, these findings demonstrate that repeated exposure to low levels of DA leads to subtle changes in VGluT1 expression within CA1 excitatory boutons, which may alter glutamatergic transmission in CA1 and disrupt behaviors dependent on spatial memory.

## 1. Introduction

Domoic acid (DA) is a neurotoxin produced by several species of marine algae, including diatoms of the genus *Pseudo-nitzschia* (Lefebvre and Robertson, 2010; Nijjar et al., 1991; Takemoto, 1978). Organisms such as anchovies, mussels, razor clams, and Dungeness crabs can accumulate high levels of DA during harmful algal blooms (Kumar et al., 2009; Lefebvre et al., 2001; Perl et al., 1990), thus posing a risk to humans, marine mammals, and seabirds that feed on these animals (Scholin et al., 2000; Sierra Beltrán et al., 1997; Work et al., 1993). Acute DA exposure in humans can cause amnesic shellfish poisoning, which is associated with gastrointestinal illness, seizures, confusion,

memory loss, coma, and death (Perl et al., 1990).

Because the incidence of the harmful algal blooms that lead to high levels of DA production is predicted to increase with ongoing climate change (Van Dolah, 2000), DA toxicity represents a significant threat to the health of humans and marine organisms. Although monitoring regulations are in place to reduce the risk of acute exposure in humans by prohibiting shellfish harvesting when DA levels are high ( $\geq 20 \mu\text{g DA/gram}$ ) (Mariën, 1996), the effects of chronic, low-level DA exposure (*i.e.* levels that do not cause overt signs of toxicity) ( $< 20 \mu\text{g/g}$ ) on human health are not well studied (Lefebvre and Robertson, 2010), and there are currently no guidelines for repetitive, low-level exposure. This is a concern because DA can persist in seafood; for example, razor clams

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retain low levels of DA (1–20 µg DA/gram) for up to one year after an algal bloom (Wekell et al., 1994). Thus, a better understanding of the potential subclinical impacts of chronic, DA exposure is necessary to determine the possible risks of repeated, low-level exposure in humans.

Studies suggest that the hippocampus is particularly susceptible to the toxic effects of acute DA exposure. Acute DA toxicosis leads to both acute and latent seizure activity in humans and sea lions (Cendes et al., 1995; Goldstein et al., 2008; Muha and Ramsdell, 2011). The seizure activity is associated with gross hippocampal alterations and changes in connectivity, neurological dysfunction, memory loss, and deficits in spatial task performance in humans, rodents, and sea lions following acute DA exposure (Cook et al., 2015; Perl et al., 1990; Petrie et al., 1992; Scholin et al., 2000; Strain and Tasker, 1991; Todd, 1993). At the cellular level, acute DA toxicity affects both neurons (Buckmaster et al., 2014; Cendes et al., 1995; Pulido, 2008; Teitelbaum et al., 1990), and glia (Chandrasekaran et al., 2004; Gill et al., 2008) of the hippocampus. DA can induce neuronal damage such as neuron degeneration, vacuolization, and swelling of dendritic processes (Ananth et al., 2003a,b, 2001; Appel et al., 1997a,b; Strain and Tasker, 1991; Tryphonas et al., 1990b), and DA-induced glial alterations include microglial activation, astrocyte swelling, necrosis, incidence of reactive astrocytes, and increased glial fibrillary acidic protein (GFAP) immunoreactivity in the hippocampus (Ananth et al., 2003a,b, 2001; Appel et al., 1997a,b; Pulido, 2008; Stewart et al., 1990; Strain and Tasker, 1991). Taken together, these reports illustrate the hippocampal neuropathology that follows acute exposure to high doses of DA, and raise the possibility that hippocampal cellular alterations may also occur following repeated exposure to lower levels of DA.

In addition to causing neuronal and glial alterations, DA may also impact excitatory and inhibitory neurotransmission in the hippocampus. DA is a tricarboxylic amino acid similar in structure to kainic acid, and is a potent agonist of both  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainic acid subtypes of ionotropic glutamate receptors (Hampson et al., 1992; Larm et al., 1997; Sciancalepore et al., 1990; Zaczek and Coyle, 1982). Activation of AMPA receptors promotes fast excitatory neurotransmission (Seeburg, 1993), and, in addition to promoting excitatory neurotransmission, activation of kainic acid receptors is known to modulate glutamate and  $\gamma$ -aminobutyric acid (GABA) release at hippocampal excitatory and inhibitory synapses (Frerking et al., 2001; Sihra and Rodríguez-Moreno, 2013, 2011). Both kainic acid and DA directly inhibit GABA release in the hippocampus (Cunha et al., 1997), which could decrease inhibitory drive and further contribute to the observed DA-induced increase in hippocampal neuronal firing (Debonnel et al., 1989). Prolonged exposure of hippocampal cultures to DA leads to increased neuronal burst spiking and electrophysiological silencing of fast-spiking interneurons (Hiolski et al., 2016), further suggesting that DA exposure impacts both hippocampal excitatory and inhibitory neural activity.

Chronic DA exposure may also lead to changes in the responsiveness of postsynaptic neurons to glutamate release, as *gria2a*, encoding a subunit of the AMPA receptor, is downregulated in zebrafish following two weeks of low-level DA exposure, and may be a compensatory response to increased neural activity (Hiolski et al., 2014). In addition, exposure to DA leads to changes that are suggestive of synaptogenesis, such as mossy fiber sprouting and upregulation of synaptophysin and PSD-95 expression (Pérez-Gómez and Tasker, 2014, 2013). Greater numbers of excitatory synapses could contribute to increased excitability and elevated neuron activity (Sutula and Dudek, 2007). Thus, exposure to DA may induce changes in hippocampal synaptic connectivity and disrupt mechanisms regulating neurotransmitter release.

While the neurotoxic effects of acute exposure to high levels of DA have been extensively studied, comparatively little is known about the effects of chronic, low-level DA exposure in adult rodents. A recent study has demonstrated spatial learning and memory deficits following repetitive low-dose DA administration in adult mice (Lefebvre et al., 2017). It is possible, but not known, that there are cellular changes in

the hippocampus that accompany these behavioral alterations. To address this, adult mice were exposed to low, asymptomatic doses of DA over 22 weeks, and the hippocampus was examined for glial, neuronal, and synaptic alterations. This chronic low-dose exposure regimen did not cause visible seizures, or lead to gross changes in hippocampal neuron density or astrocyte reactivity. Further, densities of excitatory and inhibitory synapses were not significantly altered following chronic, low-dose DA exposure. However, levels of vesicular glutamate transporter (VGluT1) immunoreactivity were selectively increased within glutamatergic boutons in the CA1 region of the hippocampus, suggesting a potential for increased glutamate release and elevated excitability.

## 2. Materials and methods

### 2.1. Animals

Adult female mice (C57Bl/6NJ) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed under standard caging conditions with *ad libitum* access to food and water. Mice included in the present study underwent repeated, low-dose DA exposure as described previously (Lefebvre et al., 2017). Briefly, mice received intraperitoneal injections of 0.75  $\pm$  0.05 µg DA/g total body weight or an equivalent volume of phosphate buffered saline (PBS) once per week for 22 weeks beginning at 12 weeks of age. No signs of acute toxicity (*i.e.* scratching, seizures, or convulsive activity) were observed immediately following DA injections. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington.

### 2.2. Tissue processing and immunohistochemistry

Following 22 weeks of DA exposure (Lefebvre et al., 2017), mice were anesthetized with 2.5% Avertin and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were extracted and post-fixed for 3–4 hours in 4% paraformaldehyde at 4 °C. After post-fixation, brains were equilibrated in 30% sucrose at 4 °C before coronal sectioning on a cryostat at a section thickness of 40 µm. Sections were stored at 4 °C in tris-buffered saline (TBS) with 0.05% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> until use in immunohistochemistry experiments.

Two hippocampus-containing sections per animal ( $n = 6$  control and  $n = 7$  DA-exposed), spaced 200 µm apart, approximately 1.7 mm posterior to Bregma, were selected for free-floating immunohistochemistry for NeuN, GFAP, or parvalbumin (PV), and cell nuclei were identified with 4',6-diamidino-2-phenylindole (DAPI). Two hippocampus-containing sections adjacent to those sections selected for cellular marker immunohistochemistry ( $n = 4$  control and  $n = 5$  DA-exposed animals) were used for free-floating immunohistochemistry for the vesicular glutamate transporters (VGluT1 and VGluT2) and the vesicular GABA transporter (VGAT) to label excitatory and inhibitory synapses. Free-floating sections were rinsed in PBS or TBS for three 10 min intervals at room temperature, and then blocked with 10% normal goat serum and 0.5% Triton-X in PBS or TBS for 2 h (cellular markers) or 1 h (synaptic markers) at room temperature. Sections were incubated at 4 °C overnight with a primary antibody against GFAP (rabbit anti-GFAP, polyclonal, Abcam (ab7260), 1:1000) or for 48 h with primary antibodies against NeuN (rabbit anti-NeuN, monoclonal, D3S3I, Cell Signaling (12,943), 1:1000) or PV (mouse anti-PV, monoclonal, Millipore (MAB1572), 1:1000) or for 96 h in primary antibodies against VGluT1 (guinea pig anti-VGluT1 (1:3000, Millipore, Billerica, MA)), VGluT2 (rabbit anti-VGluT2 (1:1000, Synaptic Systems, Goettingen, Germany)), and VGAT (mouse anti-VGAT (1:500, Synaptic Systems)). After incubation with primary antibodies, sections were rinsed three times for 10 min with PBS or TBS and incubated with secondary antibodies for 2 h at room temperature (goat anti-rabbit AlexaFluor 488 for GFAP, goat anti-rabbit AlexaFluor 647 for NeuN, and goat anti-mouse AlexaFluor 555 for PV, goat anti-guinea pig Alexa

Fluor 647 for VGluT1, goat anti-rabbit Alexa Fluor 488 for VGluT2, and goat anti-mouse Alexa Fluor 568 for VGAT (all 1:1000, Thermo Fisher Scientific). Finally, sections were washed three times for 10 min with PBS or TBS. Sections for cellular marker immunohistochemistry were incubated with DAPI for 10 min (300 nM, Invitrogen D21490/DAPI-Fluor-Pure Grade) and then washed three times for 10 min with TBS. All incubations noted were carried out on an orbital shaker. Sections were mounted on Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA) and coverslipped (Slip-Rite #1.5 coverglass, Thermo Fisher Scientific, Waltham, MA) using Fluoromount-G (Southern Biotech, Birmingham, AL). Slides were left to dry overnight prior to imaging. Sections were coded such that the experimenter was blind to exposure condition. Details of the methods and results of DAPI staining are reported in Lefebvre et al. (2017).

## 2.3. Microscopy

Sections immunostained for GFAP or NeuN were imaged on a Keyence Biorevo BZ-9000 digital widefield microscope using a 40X (0.95 N.A.) objective. For quantification of PV-immunoreactive (-IR) cells, and to measure overall hippocampal area within sections, 20X (0.75 N.A.) stacks with 2 µm spacing between Z-planes were collected capturing both hippocampal hemispheres per section and tiled to generate a map of each hippocampal hemisphere. For GFAP staining, image stacks with 1 µm steps between Z axis planes were taken at two randomly selected locations per hippocampal subregion (CA1, CA3, dentate gyrus (DG)) within each section. For NeuN and PV immunostaining, image stacks were captured at three randomly selected locations per hippocampal subregion from the more rostral of the two sections per animal. For all image stacks, imaging parameters were consistent across all stacks and sections for each antigen.

Sections stained for VGluT1, VGluT2, and VGAT immunoreactivity were imaged using a Zeiss AxioImager.M2 widefield microscope (Carl Zeiss, AG, Oberkochen, Germany), with a 63X (1.4 N.A.) Plan-Apochromat oil immersion objective, an X-Cite Series 120 mercury arc lamp (Excelitas Technologies, Waltham, MA), and a Hamamatsu Orca-ER CCD camera (Hamamatsu Photonics, Japan). Z stack collection (0.25 µm spacing between Z planes) was controlled using Stereo Investigator software (MBF Bioscience, Williston, VT). In Stereo Investigator, contours were drawn to outline the CA1, CA3 and DG regions of the hippocampus, bilaterally. The CA1, CA3, and DG regions of the hippocampus were identified using a mouse atlas (Paxinos and Franklin, 2004) and by the pattern of VGluT1 and VGluT2 immunoreactivity (Kaneko et al., 2002; Kaneko and Fujiyama, 2002). The total sampled area for each hippocampal region was not significantly different between DA-exposed and control mice (CA1:  $t_7 = 0.334$ ,  $p = 0.748$ ; CA3:  $t_7 = -1.252$ ,  $p = 0.251$ ; DG:  $t_7 = -0.328$ ,  $p = 0.752$ ). To generate random sampling sites within each region, the fractionator function was used and a square grid was randomly oriented over each region, with the X-Y spacing of the grid determined for each bilateral hippocampal region within a section according to the following formula:

$$X = \sqrt{(A_L + A_R)/5}$$

where  $X$  is the length of the grid spacing,  $A$  denotes the contour areas of the hippocampal region of interest for the left and right hemispheres of each section, and 5 is the approximate desired number of collected stacks for each region within each section. A random rotation was applied to each grid, and a counting frame was generated by Stereo Investigator at each grid intersection. At each instance where the center point of the counting frame fell within the boundary of a contour, a stack was collected. Exposure times and image collection parameters for each marker were kept constant within each hippocampal region.

## 2.4. Image processing and quantification

### 2.4.1. Cell density and immunoreactivity measurements

Image stacks collected with the 40X objective were cropped to 10 µm-thick in the Z axis, and deconvolved using AutoQuant (Media Cybernetics). The surfaces function in Imaris (v. 8.0, Bitplane, Zurich, Switzerland) was used to quantify the numbers of NeuN-IR and PV-IR cells within each image stack for each region, which were then used to calculate average cell density. GFAP- and PV-IR cell relative fluorescence intensity levels were quantified as a proxy for levels of GFAP and PV protein. To quantify GFAP labeling, GFAP-immunoreactivity for each image stack was masked using the surfaces function in Imaris, and the total sum of GFAP immunoreactivity intensity levels for each stack was determined and added across all collected stacks for each hippocampal region for each animal. The volume of GFAP immunoreactivity was similarly determined as the total sum of all GFAP-IR voxels selected by the surfaces function for each stack, added for each region within each animal. For each PV-IR cell, the sum and mean voxel intensity values and the cell volume were determined and averaged across image stacks for each region for each animal.

### 2.4.2. Synaptic marker density and fluorescence intensity measurements

Image stacks underwent 3D deconvolution using AutoQuant, with an adaptive PSF, medium noise level, and maximum of 10 iterations. Stacks were exported to Imaris and cropped to  $512 \times 512 \times 20$  pixels (approximately  $50 \times 50 \times 5 \mu\text{m}^3$ ). The surfaces function was used to identify individual VGluT1-, VGluT2-, and VGAT-IR puncta independently within each stack. It was then possible to determine the number and relative fluorescence intensity (voxel gray scale value) of immunoreactive puncta within each stack. For VGluT1 immunoreactivity, stacks were not included in the dataset if greater than 50% of the stack area encompassed the pyramidal neuron cell body layer of any hippocampal region, as there are few VGluT1-IR pre-synaptic boutons located within this layer. This reduced between-site, within-animal variability for the density measurements. Immunoreactive puncta densities were calculated for each animal based on the numbers of immunoreactive puncta within the sampled site volume, corrected for tissue shrinkage, using the following formula:

$$\text{density} = \frac{\text{number of puncta}}{(\text{sampling site number}) \times (\text{sampling site volume})} * \frac{\text{observed site Z axis thickness}}{\text{original section thickness}}$$

## 2.5. Statistical analyses

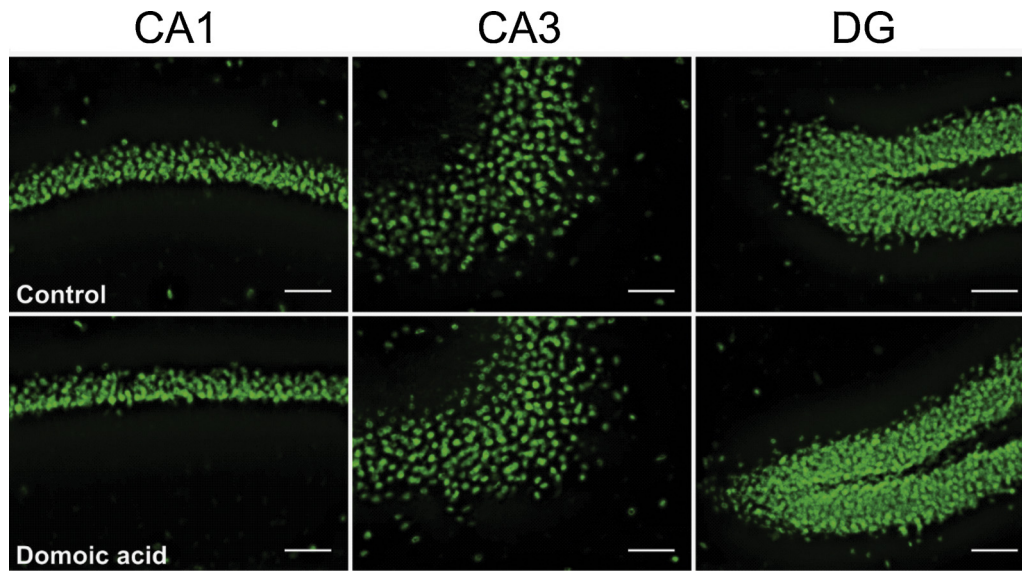
Differences in neuron densities, GFAP- and PV-immunoreactivity, immunoreactive synaptic puncta intensities and densities in each hippocampal region were analyzed using t-tests. For all tests where treatment group size was less than six, non-parametric Mann-Whitney  $U$  tests were also performed. The outcomes of the non-parametric tests did not lead to any changes of statistical significance; therefore, only results from t-tests are reported. All statistical tests were carried out at  $\alpha = 0.05$  using SPSS software (IBM, version 23).

## 3. Results

### 3.1. Chronic DA exposure does not induce overt neuronal pathology in the hippocampus

Acute DA exposure is associated with lesions and hippocampal atrophy (Ananth et al., 2001; Perl et al., 1990; Silvagni et al., 2005; Teitelbaum et al., 1990; Tryphonas et al., 1990b); however, less is known regarding the effects of chronic, low-dose exposure on the hippocampus. A previous examination of hippocampal tissue from mice





**Fig. 1.** Examples of 20X images of NeuN-IR neurons in CA1 (left), CA3 (middle), and DG (right) in control (top row) and DA-exposed (bottom row) mice. Scale bar = 50  $\mu$ m.

included in the present study did not reveal any significant alterations in hippocampal regional areas or DAPI-labeled cell density following chronic low dose DA exposure (Lefebvre et al., 2017). We measured densities of NeuN-IR cells in hippocampus to determine if chronic, low dose exposure to DA causes alterations in hippocampal neuron density. Consistent with the overall cell density observations, the densities of NeuN-IR cells were not significantly different between DA-exposed and control mice overall in the hippocampus or in any hippocampal sub-region (Fig. 1, Table 1). Together, these results demonstrate that repeated exposure to low doses of DA does not induce loss of cells, or neurons, in the hippocampus of adult mice.

Several studies have reported that acute DA exposure leads to alterations in the brain consistent with increased neuroinflammation, such as increased reactivity of astrocytes (Ananth et al., 2003b, 2001; Appel et al., 1997a,b; Kirkley et al., 2014; Pulido, 2008; Strain and Tasker, 1991; Tryphonas et al., 1990b). To assess astrocyte reactivity in a chronic, low-dose exposure model, GFAP immunoreactivity was examined (Fig. 2A and B). There were no significant changes in GFAP immunoreactivity in the hippocampus of DA-exposed mice as measured by both total intensity levels (Table 2) and average total volume of GFAP immunoreactivity within each sampled field of view (Table 2). Thus, chronic low-level DA exposure did not increase this marker of

glial reactivity, in contrast to what is observed following acute exposure. Taken together, the lack of significant changes in hippocampal area, cell and neuron densities, and lack of any significant alteration in a marker for reactive astrocytes suggests that chronic, low-dose DA exposure during adulthood does not lead to extreme neurotoxicity as might occur following an acute, symptomatic exposure event. This indicates that this repetitive, low-dose DA exposure model allows for the examination of hippocampal changes that occur independently of the neuronal loss and astrocytosis that can be caused by acute exposure.

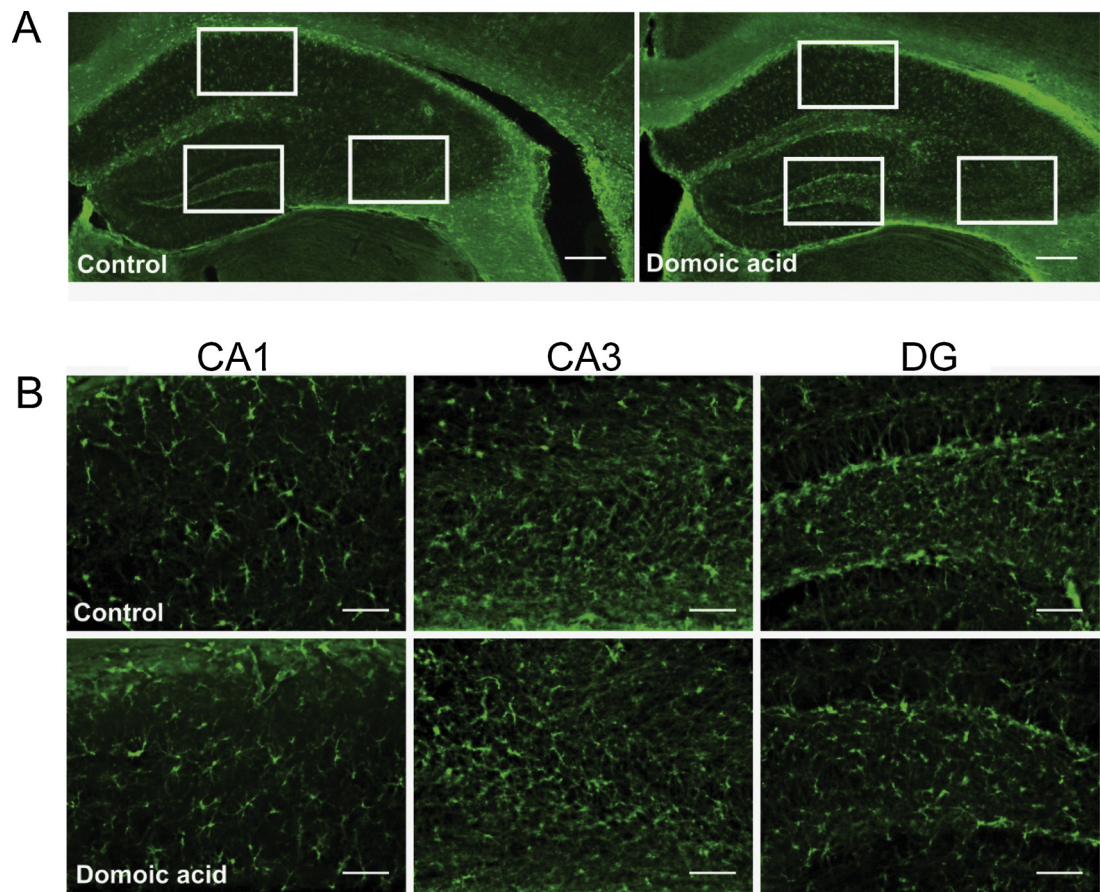
### 3.2. Chronic DA exposure leads to increased VGluT1 immunoreactivity in area CA1

VGluT1 is the vesicular glutamate transporter expressed within a population of excitatory presynaptic boutons in the brain, including the hippocampus (Freneau et al., 2004b, 2001), and changes in levels of vesicular transporters can alter the amount of neurotransmitter released from the bouton (Daniels et al., 2006, 2004; Wilson et al., 2005; Wojcik et al., 2004). Given this, if low-level DA exposure affects the levels of VGluT1 within hippocampal excitatory boutons, then glutamate release could be affected. As a proxy for VGluT1 protein content within individual VGluT1-expressing boutons, quantitative fluorescent immunohistochemistry was used to detect VGluT1 protein in hippocampal sections (Fig. 3A) and the average and total pixel fluorescence intensities within VGluT1-IR puncta of control and DA-exposed mice were measured. The mean puncta fluorescence intensity of VGluT1-IR puncta (corresponding to the average voxel gray scale value of each VGluT1-IR surface object, averaged across VGluT1-IR surface objects) was significantly increased by 14% in the CA1 region of mice exposed to DA (control: 1371.45 gray scale units, DA: 1563.47 gray scale units;  $t_7 = -3.516$ ,  $p = 0.010$ ) (Fig. 3B). In contrast to CA1, there were no significant differences between DA-exposed and control mice in measures of mean puncta intensity in CA3 ( $t_7 = -0.087$ ,  $p = 0.933$ ) or in DG ( $t_7 = -1.071$ ,  $p = 0.320$ ) (Fig. 3C and D). Consistent with the puncta mean fluorescence intensity analysis, the average sum puncta VGluT1 fluorescence intensity, which is indicative of the total amount of VGluT1 per bouton (corresponding to the sum of all voxel gray scale values for each VGluT1-IR surface object, averaged across VGluT1-IR surface objects), was significantly increased by 15% in CA1 of DA-exposed mice (Table 3). However, there were no significant differences in the average sum puncta fluorescence intensity between DA-exposed and control

**Table 1**

NeuN-immunoreactive neuron densities for individual and combined hippocampal subregions for control and DA-exposed mice. N = 6 control and 7 DA-exposed mice per group.

Measure	Control Mean (SEM)	Domoic Acid Mean (SEM)	t Statistic	p-value
<b>NeuN-IR cell density (mm<sup>-3</sup>)</b>				
<b>Combined</b>	$1.85 \times 10^5$ ( $2.86 \times 10^3$ )	$1.84 \times 10^5$ ( $9.59 \times 10^3$ )	$t(11) = 0.090$	0.930
<b>CA1</b>	$1.38 \times 10^5$ ( $3.44 \times 10^3$ )	$1.26 \times 10^5$ ( $6.40 \times 10^3$ )	$t(11) = 1.471$	0.169
<b>CA3</b>	$1.70 \times 10^5$ ( $5.36 \times 10^3$ )	$1.77 \times 10^5$ ( $1.27 \times 10^4$ )	$t(11) = -0.503$	0.625
<b>DG</b>	$2.47 \times 10^5$ ( $7.86 \times 10^3$ )	$2.48 \times 10^5$ ( $1.79 \times 10^4$ )	$t(11) = -0.460$	0.964



**Fig. 2.** A. Examples of GFAP-IR labeling in control (left) and DA-exposed (right) hippocampus. B. Examples of 20X images of GFAP immunoreactivity in CA1 (left), CA3 (middle), and DG (right) in control (top row) and DA-exposed (bottom row). Scale bars: 200  $\mu\text{m}$  (A), 50  $\mu\text{m}$  (B).

mice in CA3 or DG.

Increases in markers for synaptogenesis have been reported following DA exposure (Pérez-Gómez and Tasker, 2014, 2013); therefore, the density of VGluT1-expressing boutons could be altered in the hippocampus of mice chronically exposed to DA. However, there were no significant differences in average VGluT1-IR bouton density (puncta number per  $\mu\text{m}^3$  tissue) in CA1, CA3 or in DG (Table 3).

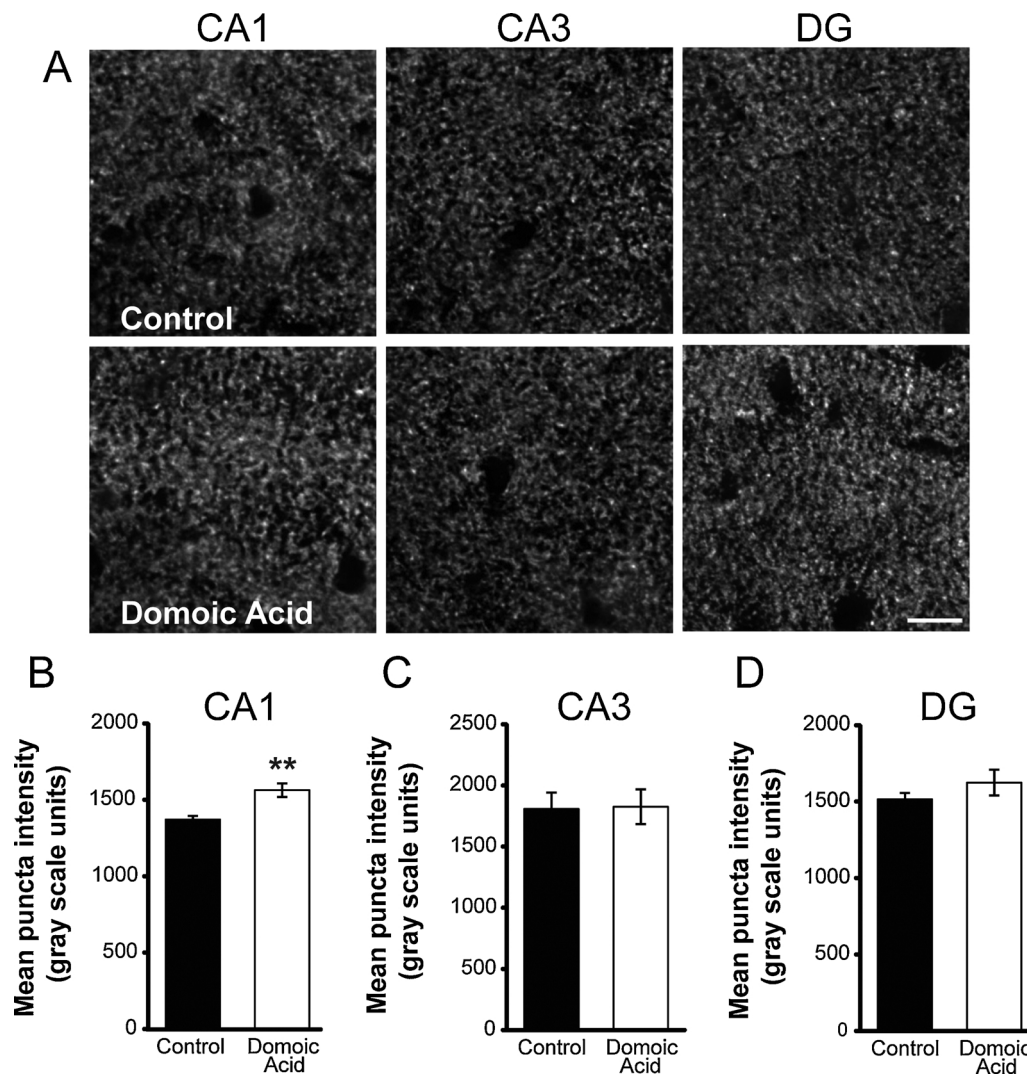
3.3. Chronic DA exposure does not affect VGluT2 immunoreactivity in DG

Excitatory boutons in the hippocampus may contain either the VGluT1 or VGluT2 isoform of the vesicular glutamate transporter, and expression of the different isoforms may reflect glutamatergic pre-synaptic boutons that arise from different origins and/or have different release properties (Freneau et al., 2004a,b; Halasy et al., 2004). Thus, bouton density and within-bouton vesicular transporter levels

corresponding to a separate and complementary population of excitatory boutons identified by the expression of VGluT2 as a vesicular glutamate transporter were also assessed. The majority of glutamatergic boutons in the hippocampus express VGluT1 rather than VGluT2, and in fact, immunoreactivity for VGluT2 is relatively low in the CA1 and CA3 regions (Freneau et al., 2001), but is present in both the granule cell and molecular layers of the DG (Freneau et al., 2001; Halasy et al., 2004; Kaneko et al., 2002; Kaneko and Fujiyama, 2002; van der Hel et al., 2009). Therefore, VGluT2 immunoreactivity was quantified only in the DG granule cell body layer and molecular layer (Fig. 4). There was no significant effect of DA exposure on VGluT2-IR puncta mean fluorescence intensity in either the granule cell body layer or the molecular layer (Table 3). Similarly, there were no significant differences in average puncta sum fluorescence intensity in either the granule cell layer or the molecular layer between DA-exposed and control mice (Table 3), nor were there significant treatment differences in puncta

**Table 2**  
Mean fluorescence intensity and volume of GFAP immunoreactivity in hippocampal subregions of control and DA-exposed mice. N = 6 control and 7 DA-exposed mice per group.

Measure	Control Mean (SEM)	Domoic Acid Mean (SEM)	t Statistic	p-value
<b>GFAP intensity</b> (gray scale units)				
CA1	$4.44 \times 10^7$ ( $5.24 \times 10^6$ )	$3.98 \times 10^7$ ( $4.97 \times 10^6$ )	$t(11) = 0.622$	0.546
CA3	$2.37 \times 10^7$ ( $2.58 \times 10^6$ )	$2.70 \times 10^7$ ( $5.38 \times 10^6$ )	$t(11) = -0.526$	0.609
DG	$3.95 \times 10^7$ ( $3.75 \times 10^6$ )	$5.63 \times 10^7$ ( $1.03 \times 10^7$ )	$t(11) = -1.442$	0.177
<b>GFAP volume</b> ( $\mu\text{m}^3$ )				
CA1	$5.17 \times 10^4$ ( $5.07 \times 10^3$ )	$4.82 \times 10^4$ ( $5.18 \times 10^3$ )	$t(11) = 0.483$	0.638
CA3	$3.17 \times 10^4$ ( $3.37 \times 10^3$ )	$3.56 \times 10^4$ ( $6.41 \times 10^3$ )	$t(11) = -0.523$	0.611
DG	$5.18 \times 10^4$ ( $4.17 \times 10^3$ )	$6.64 \times 10^4$ ( $9.66 \times 10^3$ )	$t(11) = -1.306$	0.218



**Fig. 3.** A. Examples of VGLUT1-IR puncta in CA1 (left), CA3 (middle), and DG (right) of control (top row) and DA-exposed (bottom row) mice. **B–D.** Mean fluorescence intensity of VGLUT1-IR puncta was significantly increased in CA1 in DA-exposed mice relative to controls ( $p = 0.01$ ), but not in CA3 or DG ( $p > 0.05$ ). Graphs are  $\pm$  SEM,  $n = 4$  control and  $n = 5$  DA-exposed mice. Scale bar =  $10\mu\text{m}$ .

**Table 3**

Excitatory bouton measures for control and DA-exposed mice in hippocampal subregions. \* $p < 0.05$ .  $N = 4$  control and 5 DA-exposed mice per group.

Measure	Control Mean (SEM)	Domoic Acid Mean (SEM)	t Statistic	p-value
<b>VGLUT1-IR puncta sum intensity (gray scale units)</b>				
CA1	$3.66 \times 10^5$ ( $1.42 \times 10^4$ )	$4.21 \times 10^5$ ( $9.56 \times 10^3$ )	$t(7) = -3.389$	0.012*
CA3	$4.81 \times 10^5$ ( $3.35 \times 10^4$ )	$5.04 \times 10^5$ ( $3.65 \times 10^4$ )	$t(7) = -0.439$	0.674
DG	$3.90 \times 10^5$ ( $1.53 \times 10^4$ )	$4.11 \times 10^5$ ( $2.44 \times 10^4$ )	$t(7) = -0.696$	0.509
<b>VGLUT1-IR puncta density (<math>\mu\text{m}^{-3}</math>)</b>				
CA1	0.084 (0.002)	0.086 (0.004)	$t(7) = -0.422$	0.686
CA3	0.068 (0.001)	0.067 (0.003)	$t(7) = 0.377$	0.746
DG	0.087 (0.001)	0.091 (0.004)	$t(7) = -0.760$	0.472
<b>VGLUT2-IR puncta mean intensity (gray scale units)</b>				
DG granule cell layer	2495 (215)	3147 (505)	$t(7) = -1.473$	0.184
DG molecular layer	1697 (83)	1716 (156)	$t(7) = -0.104$	0.920
<b>VGLUT2-IR puncta sum intensity (gray scale units)</b>				
DG granule cell layer	$5.14 \times 10^5$ ( $4.95 \times 10^4$ )	$6.50 \times 10^5$ ( $9.76 \times 10^4$ )	$t(7) = -1.533$	0.169
DG molecular layer	$2.39 \times 10^5$ ( $1.13 \times 10^4$ )	$2.49 \times 10^5$ ( $2.34 \times 10^4$ )	$t(7) = -0.371$	0.742
<b>VGLUT2-IR puncta density (<math>\mu\text{m}^{-3}</math>)</b>				
DG granule cell layer	0.074 (0.004)	0.069 (0.002)	$t(7) = 0.699$	0.507
DG molecular layer	0.173 (0.005)	0.168 (0.009)	$t(7) = 0.442$	0.671



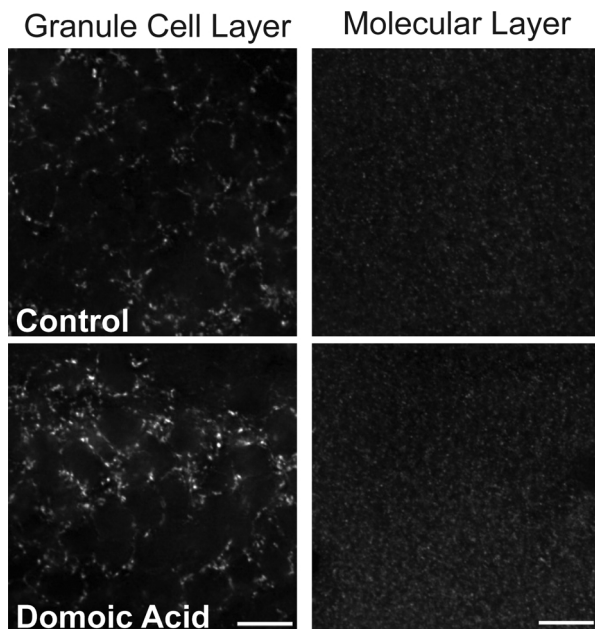


Fig. 4. Examples of VGluT2-IR puncta in the granule cell body layer (left) and the molecular layer (right) of the DG in control (top row) and DA-exposed (bottom row) mice. Scale bar = 10  $\mu$ m.

density in the granule cell body layer of the DG or in the molecular layer (Table 3).

#### 3.4. Chronic DA exposure does not affect PV or VGAT immunoreactivity in hippocampus

Studies have shown that GABAergic neurons are present in all hippocampal regions, dispersed throughout pyramidal cell layers and dendritic regions, and mainly form local intrinsic connections (Ribak et al., 1978; Storm-Mathisen et al., 1983). Subtle disruptions in GABAergic signaling can lead to hyperexcitability and hippocampal dysfunction (Thompson, 1994). In a rat model of early postnatal DA-induced seizures, PV immunoreactivity in hippocampus is decreased (Gill et al., 2010), raising the possibility that the PV subtype of GABAergic interneurons are affected by DA exposure. Further, it has recently been shown that prolonged exposure of hippocampal slice cultures to DA leads to electrophysiological silencing of fast-spiking neurons, likely corresponding to the PV-expressing population of inhibitory interneurons (Hiolski et al., 2016). Thus, it is possible that chronic, low-dose exposure to DA leads to alterations in the PV-expressing population of inhibitory neurons in the hippocampus (Fig. 5A, B). The densities of PV-IR neurons were not significantly different between DA-exposed and control mice in the combined regions of the hippocampus, or within any hippocampal subregion (Table 4). Further, levels of PV immunoreactivity within PV-IR cells were examined, as PV functions as a calcium binding protein and PV levels may impact cell excitability (Caillard et al., 2000). The average PV-IR neuron intensities were not significantly different between DA-exposed and control mice within any hippocampal subregion examined (Table 4). In addition, the average PV-IR neuron sum fluorescence intensity was not significantly different between DA-exposed and control mice, nor was the volume of PV-IR neurons different between DA-exposed and control mice (Table 4). Taken together, these results suggest that densities, size, and somal PV content of the PV-expressing population of inhibitory interneurons in the hippocampus were not impacted by chronic, low-dose exposure to DA.

Increases in neural activity have been shown to decrease levels of vesicular inhibitory amino acid transporter, suggesting that changes in excitatory and inhibitory vesicular neurotransmitter transporters may

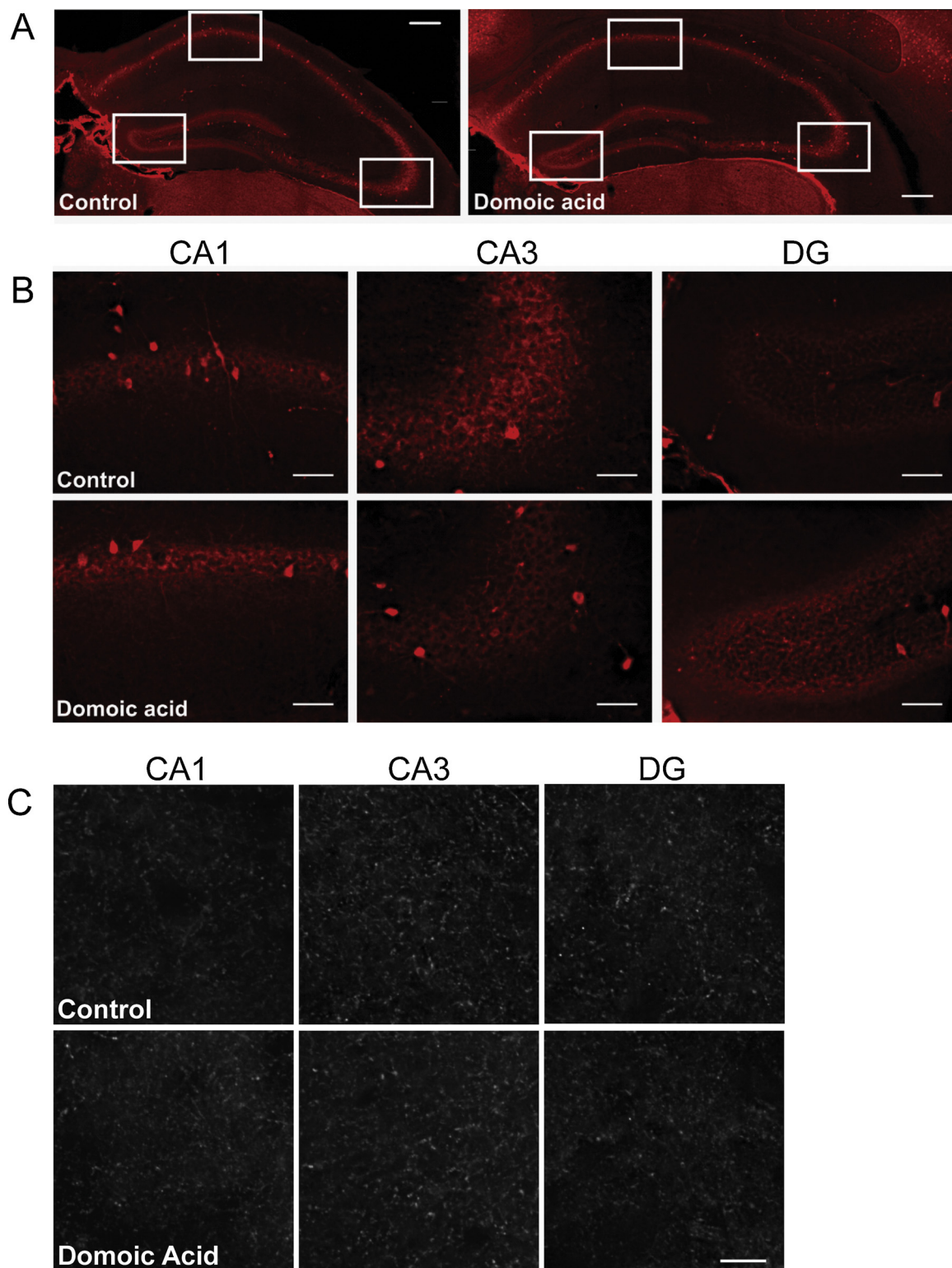
be regulated in opposite directions (De Gois et al., 2005). As DA exposure can impact excitatory activity, it is possible that homeostatic changes in the levels of the vesicular GABA transporter within inhibitory boutons, and the density of inhibitory boutons, (Fig. 5C) are present in the hippocampus of mice chronically exposed to DA. However, there were no significant differences in VGAT-IR puncta mean intensity levels, sum intensity levels, or densities between DA-exposed and control mice in CA1 (Table 4). Together with the PV-IR neuron results, this suggests that chronic, low-level DA exposure does not impact these features of inhibitory circuits in the hippocampus.

#### 4. Discussion

As climate change and other factors lead to more frequent occurrences of conditions favorable for harmful algal blooms, it is increasingly necessary to understand the structural and functional impacts of long-term exposure to neurotoxins produced by harmful algal bloom species. This is especially important given that these toxins persist at low levels for extended periods in food sources such as razor clams, increasing the risk for periodic sub-clinical exposure in humans. Recent reports suggest a link between potential exposures to chronic sub-clinical DA and learning and memory in human and mouse models (Grattan et al., 2016; Lefebvre et al., 2017). In order to determine the effects of repeated, low-dose DA exposure on cells and synapses of the hippocampus, a brain structure critical for learning and memory, cell and synaptic markers were quantified in hippocampal tissue from adult mice exposed to DA on a weekly basis for 22 weeks. VGluT1-expressing presynaptic boutons in CA1 exhibited increased VGluT1 immunoreactivity in DA-exposed mice, while densities of neurons and synaptic boutons were not altered. This effect was specific for the CA1 region, and also specific for VGluT1-IR excitatory boutons, as VGluT2-IR boutons in DG, and VGAT-IR inhibitory boutons in CA1, CA3, and DG were not significantly altered. The increase in VGluT1 levels within excitatory boutons in CA1 suggests there may be increased excitatory neurotransmission in the CA1 region of the hippocampus following chronic, low-level DA exposure. Moreover, there was no significant neuronal loss or increased GFAP expression identified in any hippocampal region. Together, these findings suggest that this model of repeated low-level DA exposure appears to induce selective changes in VGluT1 levels in CA1, in the absence of the severe neuropathology associated with acute DA exposure. In a complimentary study using a similar dosing regimen, significant learning deficits were observed in mice following 25 weeks of chronic, low-dose DA exposure, but learning returned to control levels after a nine week recovery period of no exposure (Lefebvre et al., 2017). Changes in vesicular glutamate transporter expression with chronic low-level exposure as opposed to the permanent hippocampal damage and cell loss associated with acute high level DA exposure may provide a mechanistic explanation for the reversible learning deficits observed. Whether or not the increase in VGluT1 levels is reversible upon discontinuation of chronic DA exposure will be tested in future studies.

##### 4.1. Repeated, low-dose DA exposure does not cause the same hippocampal deficits as acute, symptomatic DA exposure

These results suggest that, despite previously reported learning deficits, chronic exposure to low levels of DA does not induce severe pathological changes in the hippocampus, in contrast to acute symptomatic DA exposure. A recent study demonstrated that this DA exposure paradigm is not associated with overall hippocampal atrophy or cell loss (Lefebvre et al., 2017), consistent with the findings from other studies of low-dose DA exposure in early postnatal and adult rats (Bernard et al., 2007; Schwarz et al., 2014). Additionally, we found no significant increase in GFAP immunoreactivity, suggesting that chronic, low level DA exposure does not induce activation of astrocytes in the hippocampus. Similar to these findings, in both rats and monkeys,



**Fig. 5.** A. Example of PV immunoreactivity in the hippocampus of control (left) and DA-exposed (right) mice. B. Example images of PV immunoreactivity in CA1 (left), CA3 (middle), and DG (right) in control (top row) and DA-exposed mice (bottom row). C. Examples of VGAT-IR puncta in CA1 (left), CA3 (middle), DG (right) in control (top row) and DA-exposed mice (bottom row). Scale bar: 200 μm (A), 50 μm (B), 10 μm (C).

repeated low-dose oral exposure does not induce gross alterations in hippocampus, or changes in GFAP immunoreactivity (Truelove et al., 1997, 1996). In contrast with the findings from the chronic low-dose model used in the present study, acute doses of DA are known to elicit an increase in astrocytosis markers, including increased GFAP immunoreactivity and morphological evidence of reactive gliosis (Appel

et al., 1997a,b; Chandrasekaran et al., 2004; Ross et al., 2000; Stewart et al., 1990; Tryphonas et al., 1990a,b). Further, acute exposure of rodents to high levels of domoic acid that elicit neurological symptoms is associated with hippocampal neuronal loss and degeneration, particularly in regions CA1-CA3 (Iverson et al., 1989; Peng et al., 1994). Taken together, results from this study support the idea that effects of



**Table 4**

PV-immunoreactive neuron and VGAT-immunoreactive bouton measures in hippocampal subregions of control and DA-exposed mice. PV-IR measures: n = 6 control and 7 DA-exposed per group; VGAT-IR measures: n = 4 control and 5 DA-exposed per group.

Measure	Control Mean (SEM)	Domoic Acid Mean (SEM)	t Statistic	p-value
<b>PV-IR cell density (<math>\text{mm}^{-3}</math>)</b>				
Combined	5395 (375)	5572 (330)	t(11) = -0.355	0.729
CA1	6969 (716)	6359 (656)	t(11) = 0.629	0.542
CA3	6632 (834)	7515 (606)	t(11) = -0.874	0.401
DG	2585 (355)	2842 (145)	t(11) = -0.709	0.493
<b>PV-IR mean intensity (gray scale units)</b>				
Combined	16.90 (1.41)	16.40 (1.43)	t(11) = 0.247	0.810
CA1	16.17 (1.71)	18.53 (1.96)	t(11) = -0.893	0.391
CA3	13.01 (1.09)	12.02 (0.94)	t(11) = 0.692	0.503
DG	21.51 (2.10)	18.63 (2.11)	t(11) = 0.961	0.357
<b>PV-IR sum intensity (gray scale units)</b>				
Combined	$2.74 \times 10^5$ ( $3.29 \times 10^4$ )	$2.64 \times 10^5$ ( $2.58 \times 10^4$ )	t(11) = 0.240	0.814
CA1	$2.28 \times 10^5$ ( $2.66 \times 10^4$ )	$2.66 \times 10^5$ ( $3.71 \times 10^4$ )	t(11) = -0.812	0.434
CA3	$2.13 \times 10^5$ ( $2.34 \times 10^4$ )	$2.04 \times 10^5$ ( $2.18 \times 10^4$ )	t(11) = 0.270	0.792
DG	$3.80 \times 10^5$ ( $6.11 \times 10^4$ )	$3.21 \times 10^5$ ( $4.29 \times 10^4$ )	t(11) = 0.813	0.433
<b>PV-IR cell volume (<math>\mu\text{m}^3</math>)</b>				
Combined	1128.86 (50.06)	1158.71 (53.39)	t(11) = -0.403	0.695
CA1	1008.66 (44.70)	997.62 (50.299)	t(11) = 0.161	0.875
CA3	1150.78 (55.29)	1223.90 (69.91)	t(11) = -0.8	0.441
DG	1227.16 (83.81)	1254.60 (117.59)	t(11) = -0.184	0.857
<b>VGAT-IR puncta mean intensity (gray scale units)</b>				
CA1	1421 (83)	1457 (93)	t(7) = -0.280	0.788
CA3	1753 (130)	1783 (149)	t(7) = -0.151	0.884
DG	1286 (56)	1374 (83)	t(7) = -0.825	0.437
<b>VGAT-IR puncta sum intensity (gray scale units)</b>				
CA1	$2.47 \times 10^5$ ( $3.06 \times 10^4$ )	$2.66 \times 10^5$ ( $2.03 \times 10^4$ )	t(7) = 0.539	0.606
CA3	$3.54 \times 10^5$ ( $3.41 \times 10^4$ )	$3.48 \times 10^5$ ( $3.30 \times 10^4$ )	t(7) = 0.119	0.909
DG	$2.46 \times 10^5$ ( $1.38 \times 10^4$ )	$2.57 \times 10^5$ ( $1.83 \times 10^4$ )	t(7) = -0.438	0.677
<b>VGAT-IR puncta density (<math>\mu\text{m}^{-3}</math>)</b>				
CA1	0.171 (0.019)	0.174 (0.011)	t(7) = -0.153	0.882
CA3	0.139 (0.015)	0.138 (0.010)	t(7) = 0.056	0.957
DG	0.221 (0.015)	0.193 (0.012)	t(7) = 1.46	0.187

repeated, low-dose DA exposure on hippocampal neurons and astrocytes differ from those effects induced by acute exposure to higher doses of DA. Future work should be done to assess the effects of repeated low-dose domoic acid exposure specifically on activation of microglia, glial cells that also contribute to neuroinflammatory responses, and have been suggested to play a role in the neurotoxic effects of DA (Ananth et al., 2001; Appel et al., 1997a,b; Chandrasekaran et al., 2004).

A number of studies of acute DA exposure have reported a range of effects on the GABA system. GABAergic neurons participate in feedback and feedforward inhibitory circuits in the hippocampus (Kullmann, 2011), and changes in the number or function of GABAergic synapses are likely to impact pyramidal neuron firing and hippocampal function (Hájos and Paulsen, 2009; Mann et al., 2005). In chick retina cells, application of DA stimulates GABA release (Alfonso et al., 1994), while it inhibits GABA release from hippocampal synaptosomes via actions at presynaptic kainic acid receptors (Cunha et al., 1997). In adolescent mice that received an acute *in utero* exposure to DA, levels of GABA in the hippocampus and cortex are significantly reduced (Dakshinamurti et al., 1993). However, another study found that an acute dose of DA given to adult rats increases GABA levels in the hippocampus, cortex, and amygdala, which could indicate increased GABA production and/or release following acute DA exposure (Durán et al., 1995). In contrast with reports of acute DA exposure-induced effects on GABAergic neurons, there was no significant effect of chronic, low-level DA exposure on the density or VGAT content of hippocampal GABAergic boutons, or the densities or PV content of PV-IR neurons. The findings of the present study differ from a low-dose, early postnatal DA-induced seizure model in rat, where PV immunoreactivity was decreased in the DG region of the dorsal hippocampus (Gill et al., 2010). It is possible that this discrepancy could indicate that the developmental timing of low-dose DA

exposure is crucial, and earlier (postnatal) exposure may cause alterations in PV-IR hippocampal interneurons that are not observed following adult low-dose DA exposure. A study previously found that prolonged *in vitro* DA exposure leads to electrophysiological silencing of fast-spiking inhibitory interneurons in organotypic hippocampal culture (Hiolski et al., 2016). Taken together with results of the current study, this could suggest that chronic DA exposure does not lead to physical loss of PV-expressing interneurons, but may reduce their activity. It is also possible that repeated low-dose DA exposure may impact other interneuron populations, such as somatostatin-expressing interneurons. For example, chronically-exposed sea lions have fewer hilar somatostatin-positive neurons (Buckmaster et al., 2014), although a prior study reported no significant alterations in somatostatin-positive neuron counts in hippocampus following postnatal low-dose DA exposure (Gill et al., 2010). Future studies will be needed to gain insight into chronic domoic acid-induced alterations in other hippocampal interneuron populations. However, VGAT is a marker for GABAergic boutons from all populations of GABAergic interneurons, and the results of the present study do not suggest any change in density of inhibitory boutons in hippocampus, although it is possible that there are changes in inhibitory boutons attributable to specific interneuron subpopulations. Taken together with the lack of significant changes in hippocampal cell and neuronal counts, and astrocyte reactivity, the absence of alterations in markers of GABAergic synapses further differentiates the effects of low-level chronic DA exposure during adulthood from those effects caused by acute exposure to high doses of DA.

#### 4.2. Chronic DA exposure may affect vesicular regulation of glutamatergic neurotransmission

The results of the present study suggest that chronic, low-dose DA

exposure increases the level of VGluT1 protein within presynaptic boutons in CA1 of adult mice. VGluT1 is responsible for packaging glutamate into synaptic vesicles, and the extent of vesicle filling depends in part on the number of active vesicular transporters (Wilson et al., 2005). Under normal conditions, synaptic vesicles within glutamatergic and GABAergic boutons are not consistently filled with neurotransmitter to maximum capacity (Engel et al., 2001; Yamashita et al., 2003), suggesting that increasing or decreasing the amount of neurotransmitter transported into vesicles by adjusting numbers of available vesicular transporters should subsequently increase or decrease quantal size. In support of this, studies have demonstrated that the level of VGluT1 regulates quantal release of glutamate, and alters postsynaptic responses (Daniels et al., 2006, 2004; Wilson et al., 2005; Wojcik et al., 2004). For example, VGluT1 deletion impairs glutamatergic transmission and overexpression of VGluT1 increases quantal size (Freneau et al., 2004a; Wojcik et al., 2004). In drosophila, VGluT overexpression increases mini excitatory postsynaptic potential amplitude, and increases synaptic vesicle size, suggesting that increasing VGluT expression increases the total glutamate content of each vesicle (Daniels et al., 2004). VGluT overexpression also decreases synaptic transmission failure rate, which could indicate an increase in the number of vesicle release events large enough to elicit an AMPA receptor-mediated response, or an increase in the probability that a vesicle will be released after an action potential (Herman et al., 2014; Wilson et al., 2005). Taken together, these studies indicate that increasing and decreasing levels of vesicular neurotransmitter transporters is a mechanism by which neurotransmitter release, and thereby synaptic activity, may be altered. Thus, the finding of increased within-bouton VGluT1 immunoreactivity following DA exposure may be associated with increased glutamate release in the hippocampus CA1 region. This could contribute to increased hippocampal excitability, and/or memory impairment, given that aged rats with memory deficits exhibit increased VGluT expression (Ménard et al., 2015). Interestingly, adult female mice that underwent a similar repeated, low-level DA exposure paradigm exhibit spatial memory impairment (Lefebvre et al., 2017), suggesting that this exposure paradigm may be associated with both changes in hippocampal VGluT1 levels and memory impairments.

The increase in within-bouton VGluT1 levels was observed in boutons within the CA1 region of the hippocampus, but not CA3 or DG. VGluT1-immunoreactive boutons located in CA1 represent glutamatergic excitatory inputs to the CA1 region. One source of glutamatergic input to CA1 is the Schaffer collateral projections that arise from pyramidal neurons in CA3. *In vivo* evidence demonstrates that DA is 20 fold more potent in activating the CA3 region compared to the CA1 region (Debonnel et al., 1989) and the CA3 region of the hippocampus has a high density of kainic acid receptor binding sites (Monaghan and Cotman, 1982), as well as a lower threshold for kainic acid-induced seizures (Schwartzkroin and Prince, 1978). In addition, acute contaminated mussel extract exposure or DA exposure in rats revealed alterations in the CA3 region of the hippocampus (Tryphonas et al., 1990b,c). These reports are consistent with the idea that the increased VGluT1 levels in CA1 observed following repeated low-level DA exposure may reflect changes in the glutamatergic Schaffer collateral projections from CA3 pyramidal neurons to CA1. On the other hand, it is important to note that DA also induces potent excitation of CA1 *in vivo* (Debonnel et al., 1989), and increased excitability of CA1 has been observed after DA exposure in hippocampal slices with CA3 removed (Sari and Kerr, 2001), suggesting that DA exerts effects on excitatory neurotransmission in CA1 independently of its effects on CA3. Additionally, low dose DA applied to hippocampal slice cultures induces injury to cells in CA1 to a greater extent than areas CA3 or DG (Pérez-Gómez and Tasker, 2012). It will be informative to elucidate in future studies whether the increased CA1 VGluT1 levels following repeated low-dose DA exposure are caused by direct effects on area CA1, or whether increased VGluT1 in CA1 occurs downstream of effects on other regions such as CA3.

Further studies will be necessary to investigate the behavioral implications of altered within-bouton VGluT1 levels in the CA1 region. However, neuronal activity in the CA1 region is important for spatial memory (Jo et al., 2007; Moser and Moser, 1998; Tsien et al., 1996), and output from CA3 is important for rapid acquisition of memories in novel environments and for spatial tuning in CA1 pyramidal neurons (Nakashiba et al., 2008). Therefore, if repeated DA exposure increases glutamate release at CA3 to CA1 synapses, then this may impact spatial learning and memory in DA-exposed animals. In support of this, a previous study demonstrated that a similar repeated low dose DA exposure regimen caused spatial learning and memory impairment in mice (Lefebvre et al., 2017). These behavioral impairments were no longer present two months after the cessation of DA exposure, suggesting that the DA-induced spatial behavior impairments are reversible. Future studies will be necessary to determine if increased CA1 VGluT1 levels are similarly reversible, and to determine if there is a causal relationship between excitatory synapse alterations and spatial deficits in chronic, low-level DA-exposed mice.

#### 4.3. Effects of chronic DA may be selective for changes in within-hippocampus glutamatergic boutons, rather than glutamatergic hippocampal afferents

Protein expression patterns of VGluT1 and VGluT2 are complementary in the hippocampus (Kaneko et al., 2002; Kaneko and Fujiyama, 2002), with VGluT2 immunoreactivity being much greater in DG than in CA1 or CA3. There were no significant changes in VGluT2-IR bouton fluorescence intensities or densities in DG, suggesting that the VGluT2-expressing population of excitatory boutons in DG is not affected by low-level, chronic DA exposure, and VGluT1-expressing boutons may be selectively affected. These boutons represent a subpopulation of glutamatergic boutons separate from the VGluT1-expressing bouton population in hippocampus. It has been reported that VGluT2 mRNA and protein are not expressed within hippocampal cells (Kaneko et al., 2002; Kaneko and Fujiyama, 2002; Ziegler et al., 2002), which suggests that VGluT2-IR boutons quantified in the present study may not originate within the hippocampus. Multiple studies have shown that VGluT2-expressing boutons originate outside the hippocampus (Freneau et al., 2001; Halasy et al., 2004; Herzog et al., 2001; Hisano et al., 2000), and could represent, for example, projections to the DG from the medial septum diagonal band complex and the supramammillary nucleus (Boulland et al., 2009; Freneau et al., 2001; Halasy et al., 2004; Hajszan et al., 2004; Lin et al., 2003; Ziegler et al., 2002). The fact that no alterations in VGluT2-expressing boutons were observed in the current study, while alterations in some VGluT1-expressing boutons were found, may suggest that chronic, low dose DA exposure primarily affects the hippocampus, rather than indirectly inducing changes in the hippocampus that are downstream of disruptions in other brain regions.

#### 4.4. Limitations

There are several possible limitations to the present study, including the relatively small treatment group size, and that only female animals were studied. The small treatment group size and reduced statistical power may have affected our ability to detect a DA treatment effect in some outcomes, and suggests our negative findings should be interpreted with caution. Previous work has reported effects of domoic acid in female adult animals; for example, acute doses of domoic acid lower than those necessary to elicit neurological symptoms (e.g. seizures) alter the expression of c-fos immunoreactivity in hippocampus (Peng and Ramsdell, 1996). However, during the 1987 human DA exposure event, toxic effects were reportedly more severe in males (Perl et al., 1990). In animals, evidence suggests that males could be more susceptible to severe neurotoxic effects of DA following an acute high dose (Baron et al., 2013), and male rats exhibit increased lateral septal area cell loss

compared to females after acute local DA infusion (Wetmore and Nance, 1991). Thus, it is important to consider that the effects reported here are not necessarily generalizable to males, and it will be important to determine the effects of chronic, low-level domoic acid exposure in adult male mice in future studies.

#### 4.5. Conclusion

In summary, these results demonstrate that repeated, low level DA exposure in adult mice is associated with a selective increase in vesicular glutamate transporter levels within VGluT1-expressing boutons in the CA1 region of the hippocampus that occurs in the absence of markers of gross morphologic and neuroinflammatory alterations that follow acute exposure to high DA levels. This suggests there may be increased glutamate release from presynaptic excitatory boutons in the CA1 region that could lead to increased hippocampal excitability that may contribute to the hyperactivity and spatial learning and memory deficits reported previously following a similar repeated, low-level DA exposure regime (Lefebvre et al., 2017). Future studies will determine the cellular and molecular mechanisms that contribute to increased VGluT1 expression in the CA1 region of the hippocampus following repeated low-level DA exposure, and further elucidate the impacts of increased CA1 VGluT1 levels on neurobehavioral performance.

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