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Research Article

Selective Enhancement of Domoic Acid Toxicity in Primary Cultures of Cerebellar Granule Cells by Lowering Extracellular Na^+ Concentration

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ABSTRACT

Domoic acid (DOM) is an excitatory amino acid analog of kainic acid (KA) that acts through glutamic acid (GLU) receptors, inducing a fast and potent neurotoxic response. Here, we present evidence for an enhancement of excitotoxicity following exposure of cultured cerebellar granule cells to DOM in the presence of lower than physiological Na^+ concentrations. The concentration of DOM that reduced by 50% neuronal survival was approximately 3 μM in Na^+-free conditions and 16 μM in presence of a physiological concentration of extracellular Na^+. The enhanced neurotoxic effect of DOM was fully prevented by AMPA/KA receptor antagonist, while N-methyl-D-aspartate-receptor-mediated neurotoxicity did not seem to be involved, as the absence of extracellular Na^+ failed to potentiate GLU excitotoxicity under the same experimental conditions. Lowering of extracellular Na^+ concentration to 60 mM eliminated extracellular recording of spontaneous electrophysiological activity from cultured neurons grown on a multi electrode array and prevented DOM stimulation of the electrical activity. Although changes in the extracellular Na^+ concentration did not alter the magnitude of the rapid increase in intracellular Ca^{2+} levels associated to DOM exposure, they did change significantly the contribution of voltage-sensitive calcium channels (VScaCs) and the recovery time to baseline. The prevention of Ca^{2+} influx via VSCaCs by nifedipine failed to prevent DOM toxicity at any extracellular Na^+ concentration, while the reduction of extracellular Ca^{2+} concentration ameliorated DOM toxicity only in the absence of extracellular Na^+, enhancing it in physiological conditions. Our data suggest a crucial role for extracellular Na^+ concentration in determining excitotoxicity by DOM.

Key words: cerebellar granule neurons; excitotoxicity; non-NMDA receptors.

Domoic acid (DOM) is a tricarboxylic amino acid analog of the excitatory amino acid (EAA) neurotransmitter L-GLU. It is produced by the red seaweed *Chondria armata* and by various marine diatoms of the *Pseudo-nitzschia* genus and was identified as the toxic agent responsible for an outbreak of poisoning due to consumption of contaminated shellfish (Perl *et al.*, 1990; Pulido, 2008). Neuronal loss in the amygdala and the hippocampus was observed in the individuals who died following such intoxication (Teitelbaum *et al.*, 1990), and similar brain lesions have been seen in primates, rats, and mice following DOM exposure

(Scallet *et al.*, 1993; Sobotka *et al.*, 1996; Strain and Tasker, 1991; Tryphonas *et al.*, 1990), with a pattern of brain damage similar to that observed after administration of kainic acid (KA) (Teitelbaum *et al.*, 1990). Domoic acid is indeed structurally similar to KA and has being shown to be a ligand for the non-N-methyl-D-aspartate (NMDA) group of ionotropic GLU receptors (Hampson and Manalo, 1998), and several studies have shown that it activates with high and low affinity the KA and α-amino-3-hydroxy-5-methylisoxazole-4-proponic acid (AMPA) types of EAA receptors, respectively, (Chittajallu *et al.*, 1999; Dingledine *et al.*, 1999). As such, DOM has been used as a reliable research tool to investigate excitotoxic damage induced by AMPA/KA *in vivo* (Routbort *et al.*, 1999; Ryan *et al.*, 2005, 2011; Xu *et al.*, 2008) and *in vitro* (Gill *et al.*, 2008; Novelli *et al.*, 1992; Pérez-Gómez *et al.*, 2010; Pérez-Gómez and Tasker, 2012).

Although many of the toxicological properties of DOM in mammals can be accounted for by its interaction with non-NMDA receptors (Novelli *et al.*, 1990, 1992), DOM-mediated increase in $[Ca^{2+}]i$ may result in the release of L-GLU, which in turn contribute to excitotoxicity via activation of NMDA receptors (Berman and Murray, 1997; Berman *et al.*, 2002; Fernández-Sánchez and Novelli, 1996; Giordano *et al.*, 2006; Novelli *et al.*, 1990). Sodium and Ca^{2+} are the two most important ions entering the neurons via a direct or indirect coupling to EAA receptor stimulation (Chittajallu *et al.*, 1999; Dingledine *et al.*, 1999). The role of Ca^{2+} as a second messenger in neurons is well-established and its role in excitotoxicity has been the subject of numerous studies (for review: Pulido, 2008; Novelli *et al.*, 2014). The possibility that Na^+ ions may also play a role intracellularly other than the modulation of membrane potential has received less attention. It has been shown that the absence of Na^+ may modify KA receptor tridimensional structure resulting in changes in the affinity for the extracellular binding site on the receptor (Chaudhry *et al.*, 2009). A series of experiments aimed at elucidating the channel permeability of native KA receptor concluded that KA receptor mediated current is undetectable in the absence of extracellular Na^+. In contrast, AMPA receptor-mediated responses were unaffected. This result indicated that different gating mechanisms were imposed by the presence or absence of Na^+ (Paternain, *et al.*, 2003).

In this study, we have used primary cultures of cerebellar neurons to test whether extracellular Na^+ could modulate the neurotoxicity of DOM. These glutamatergic neurons express all types of EAA receptors in culture, as well as voltage-sensitive ion channels including Ca^{2+} and Na^+ channels, and have proved very useful in the study of the biochemical events coupled to neurodegeneration by a variety of stimuli and the conditions controlling excitotoxicity (Díaz-Trelles *et al.*, 1999, 2002; Fernández-Sánchez and Novelli, 1993; Fernández-Sánchez *et al.*, 2001; Novelli *et al.*, 1988). Moreover, we have found cerebellar cultures to be a very convenient experimental model to investigate the action of different types of marine toxins, including DOM, palytoxin, yessotoxin, and the diarrheic shellfish poisoning toxins okadaic acid and dinophysistoxins (Fernández *et al.*, 1991; Fernández-Sánchez *et al.*, 1996; Novelli *et al.*, 1992; Pérez-Gómez *et al.*, 2004, 2006, 2010).

Here, we provide evidence that excitotoxicity by DOM is enhanced in cultured cerebellar neurons exposed to lower than physiological concentrations of Na^+ ions, while glutamate (GLU) excitotoxicity is unchanged. Under these conditions, we provide evidence that the enhancement of DOM toxicity is associated with Ca^{2+} permeability of AMPA receptors.

MATERIALS AND METHODS

Primary Neuronal Cultures

Primary cultures of rat cerebellar neurons and primary cultures of mouse cortical neurons were obtained from the animal colonies hosted in the Bioterium of the University of Oviedo. The animal procedures used were in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the University of Oviedo.

Cerebellar neurons. Primary cultures of rat cerebellar neurons were prepared as previously described (Novelli *et al.*, 1988). Cytosine arabinoside (10 μM) was added after 20–24 h of culture to inhibit the replication of nonneuronal cells. After 8 days *in vitro*, morphologically identifiable granule cells accounted for more than 95% of the neuronal population, the remaining 5% being essentially GABAergic neurons. Astrocytes did not exceed 3% of the overall number of cells in culture. Cerebellar neurons were kept alive for more than 40 days in culture by replenishing the growth medium with glucose every 4 days and compensating for evaporative losses.

Cortical neurons. Primary cultures of cortical neurons were prepared from CD1 mice optimizing the protocol described by Novelli *et al.* (1988) with some modification (Ahlemeyer and Baumgart-Vogt, 2005; Hales *et al.*, 2010; Hilgenberg and Smith, 2007; Kivell *et al.*, 2000). Briefly, the brains from 0 to 2 day postnatal pups were extracted from the skull and the cerebellum was excised. Then, the brains were sliced and the cortex was dissected from each slice and processed both enzymatically and mechanically to obtain a cell suspension that was resuspended in Neurobasal A growth medium B27 supplemented with 1% B27, 2 mM L-glutamine, and 100 μg/ml gentamicin (NBMS) and distributed to multi electrode array (MEA) wells to achieve an initial density of 6000 cells/mm^2. For maintenance, 25% of the initial NBMS was substituted with fresh NBMS every 3 days after the first week.

Neurotoxicology

Neurons were used between 14 and 20 days in culture. Cells were washed twice and incubated at 37°C in a buffer solution (modified Locke's solution [LS]) during the experiments, containing (in mM): 130 NaCl, 5.6 KCl, 5.6 glucose, 8.6 HEPES, 1 $MgCl_2$, and 2.3 $CaCl_2$ (pH 7.4). The above LS was used as the Na^+-containing solution [+NaLS]. Na^+-free solution [−NaLS] contained 130 mM choline chloride and no added NaCl. Mg^{2+}-free solution (−Mg+NaLS) contained 1 mM choline chloride and no added $MgCl_2$. $Mg^{2+}$and Na^+ free solution (−Mg−NaLS) contained 131 mM choline chloride and added neither NaCl nor $MgCl_2$. Drugs were added into the corresponding buffer solution at the indicated concentrations, and neuronal cultures were observed for signs of neurotoxicity thereafter by phase contrast microscopy at the specified times. To quantify neuronal survival after 9 h exposure to drugs, cultures were stained with fluorescein diacetate and ethidium bromide (Fernández *et al.*, 1991; Novelli *et al.*, 1988). Representative photographs of at least 3 randomly selected culture fields (n) for each experimental condition were obtained and live and dead neurons were counted. In untreated cultures, each field contained ≈500 neurons. Results are expressed as percentage of live neurons. All the experiments have been performed in duplicate and repeated at least twice.

Confocal Microscopy

For the determination of the intracellular concentration of Ca^{2+}, neuronal cultures were loaded for 20–30 min with 5 μM Fluo-3-AM ester in the incubation buffer with the corresponding Na^+ concentration. The dye was then removed by washing the culture with fresh buffer of the same Na^+ concentration, and cultures were allowed to reequilibrate for 15 min before recording either in the presence or in the absence of voltage-sensitive calcium channels (VSCaCs) blockers. Fluo-3 emission (> 515 nm) was recorded in a Leica TCS-SP2-AOBS confocal microscope with a krypton-argon laser excitation source (488 nm), and signals were digitized using Leica Confocal software and analyzed by ImageJ (1.37 k). Fluorescence intensity was quantified in at least 10–15 cell bodies (*n*) per field and all the experiments have been repeated at least twice.

Electrophysiology

Extracellular recording of neuronal spontaneous activity was obtained from cultured cortical neurons grown in a MEA device that fitted the USB-MEA-System from Multi Channel Systems (Reutlingen, Germany). Standard MEAs from Multi Channel System (60MEA200/30iR-Ti-gr consisting of 60 titanium planar electrodes embedded in a glass slide) were used, with 30 μm electrode diameter spaced by a 200 μm distance. Raw analog signals were sampled at 25 kHz and, after amplification (bandwidth 1 Hz–3 kHz), were filtered with a high-pass band filter (Butterworth second order) at 200 Hz (Mack *et al.*, 2014) to avoid local field potential that settle in the low frequency range (Belitski *et al.*, 2008), and attain higher signal-to-noise ratio and lower false positive rates (Obien *et al.*, 2015). Recordings were performed on 12–24 day-*in vitro* mouse cortical neurons cultured on MEA dishes in which activity is fairly stable (Puia *et al.*, 2012). Only MEAs with at least 10–20 active electrodes (*n*) were taken into account for analysis (Novellino *et al.*, 2011), and all the experiments have been repeated at least twice. No detectable spontaneous electrical activity could be recorded from cerebellar neurons in any culturing condition.

Chemicals

Domoic acid and 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) were from Tocris bioscience. Fluo-3-AM was from Molecular Probes. All other chemicals were from Sigma.

Data Presentation and Analysis

Graphs and curve fits were obtained using Sigma Plot version 10. For statistical analysis, we have used the software InStat for Macintosh, version 2.01. An unpaired two tailed one way ANOVA or a student *t* test was used to identify overall significant differences. A Tukey-Kramer multiple comparison test was used for selective comparison of individual data groups. Homogeneity of variances was tested using a Bartlett's test. Reported values are the mean ± SD/SEM of the number of items (*n*) specified in each method. Only significances relevant for the discussion of the data are indicated in each figure.

RESULTS

Exposure to Na^+-Free Conditions Enhances Excitotoxicity by DOM But Not by Glutamate in Cultured Cerebellar Neurons

We first examined the effects of neuronal exposure to Na^+-free conditions on neuronal survival. Na^+ concentration in the incubation buffer was reduced from 130 mM (+NaLS) to 0 mM by iso-osmotic substitution of Na^+ with choline (−NaLS) (see Materials and Methods). Under such conditions, neither morphological signs of toxicity nor significant changes in cell survival could be observed for up to 10 h as compared with neurons incubated in presence of a physiological concentration of Na^+.

Exposure of cultured cerebellar neurons to concentrations of DOM that did not compromise neuronal viability when incubated with +NaLS significantly reduced neuronal survival in absence of Na^+ (Figure 1). Dose-response experiments were performed using neuronal retention of the vital dye fluorescein as a marker of neuronal integrity and survival (Figure 1A). The concentration of DOM that produced a 50% reduction in neuronal survival (EC50) after a 9 h incubation in −NaLS was estimated at approximately 3 μM (Figure 1B). In the absence of Na^+, neurotoxicity was significantly increased above DOM concentrations of 1 μM and 90% neurotoxicity was observed following exposure to 5 μM DOM while no significant reduction in neuronal survival was observed in cultures exposed to the same concentration of DOM in +NaLS (Figure 1B).

Neurodegeneration by DOM was characterized by: (1) early swelling and darkening of the cell body (5–30 min); (2) progressive neurite network fragmentation (4–9 h); and (3) cell body disintegration (6–9 h). These common features were observed when toxic concentrations of DOM were added to the buffer either in the presence or absence of sodium (Figure 2A). Although the large majority of the neurons in culture exposed to toxic concentrations of DOM underwent the first step quite rapidly, the disintegration of the cell body followed a slower time course (Figure 2A). Thus, exposure to 5 μM DOM in −NaLS for 6 h reduced the number of vital neurons by 50%, while 9 h exposure to the toxin was necessary to reduce the number of vital neurons by 90% (Figure 2B). Neither visible morphological signs of toxicity nor changes in the number of vital neurons was observed in cultures exposed to 5 μM DOM for up to 9 h in the +NaLS. For comparison, the concentration of DOM reducing neuronal survival by 90% increased from 5 μM in −NaLS to 25 μM in +NaLS (Figs. 1B and 2B) while the lowest concentration of DOM reducing neuronal survival and the EC50 in +NaLS were 7 and 16 μM, respectively, (data not shown). To determine the minimum concentration of extracellular Na^+ necessary to prevent the enhancement of DOM neurotoxicity, neuronal cultures were incubated in LS containing increasing concentrations of Na^+ (0, 20, 40, 60, 80, and 130 mM) and then exposed to DOM (5 μM) for 9 h. A slight DOM potentiation was detected in neurons exposed to 60 mM Na^+, although concentrations as low as 40 mM Na^+ were necessary to observe a significant decrease in neuronal survival after a 9 h incubation with DOM 5 μM (Figure 3).

Next, we explored the possibility that extracellular Na^+ reduction could also modify GLU neurotoxicity. Neuronal cultures incubated either in +NaLS or −NaLS and exposed to increasing concentrations of GLU for up to 9 h did not show signs of toxicity, either at early or at later times following GLU exposure at concentrations up to 1 mM (Table 1). Removal of Mg^{2+} from either incubation condition produced a similar concentration-dependent neurotoxicity with approximately 55% and 90% of neuronal death at 500 μM and 1 mM, respectively, in the presence or absence of sodium (Table 1).

Enhancement of Neurotoxicity by DOM Is AMPA/KA receptor Dependent

To investigate whether the extracellular concentration of Na^+ could modify the receptor pharmacology of DOM, we first used

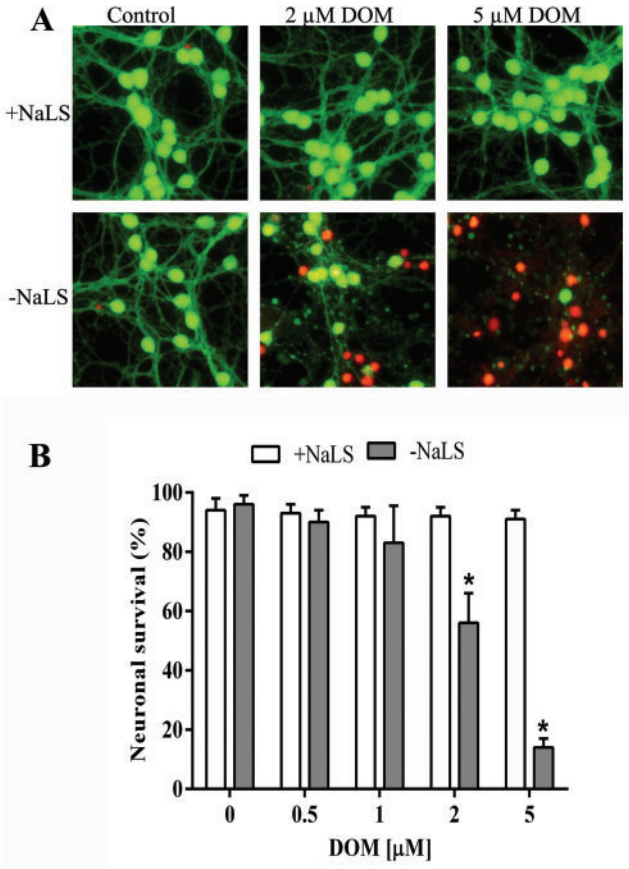

Figure 1. Enhancement of domoic acid (DOM) toxicity in the absence of extracellular Na^+. Cultured cerebellar neurons at 14–20 days in culture were incubated in a modified Locke's solution (LS) as described in Materials and Methods, containing either a physiological concentration of Na^+ (130 mM NaCL, +NaLS) or no Na^+ (0 mM NaCl, −NaLS). After 15 min, DOM was added at the indicated concentrations and neuronal survival was determined after 9 h exposure to this drug. A, Fluorescein diacetate/ethidium bromide staining of cultures following exposure to the indicated treatments: live neurons retain fluorescein in the cell body and the neurites, while ethidium bromide labels the nuclei of dead neurons; B, Quantification of the results presented in (A): enhancement of DOM neurotoxicity in the absence of extracellular Na^+ in comparison with the effects of the same concentration of DOM in the presence of 130 mM extracellular Na^+. Data are the mean ± SD ($n = 3$–6 neuronal fields). *$p \leq .01$.

two non-NMDA receptor antagonists: 4-(8-methyl-9H-1, 3-dioxolo[4, 5-h][2, 3]benzodiazepin-5-y l)-benzenamine hydrochloride, (GYKI, 50 μM), a relatively selective AMPA receptor antagonists (Jakus *et al.*, 2004) and the broader spectrum AMPA/KA antagonist CNQX (15 μM). Domoic acid toxicity was effectively prevented by the presence of either GYKI or CNQX at any extracellular concentration of Na^+ tested (Figure 3).

We then investigated whether NMDA receptors play any role in the neurodegenerative process promoted by DOM in LS. Thus, we examined whether the presence of the NMDA receptor antagonist (5R, 10S)-(+)-5-methyl-10, 11-dihydro-5H-dibenzo[a, d]cyclohepten-5, 10-imine maleate (MK-801) (1 μM) may reduce any of the *three* DOM-induced toxicity features previously described. Neuronal culture incubated in either +NaLS or −NaLS and exposed to DOM at a concentration of 25 and 5 μM, respectively, in the presence or absence of MK-801. No reduction in the toxicity was observed in any of the three toxicity features following DOM exposure (Table 2).

Extracellular Na^+-Dependent Kinetics of Intracellular Ca^{2+} Concentration following Exposure to DOM

We then studied whether changes in extracellular Na^+ concentration may alter intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) both before and after DOM exposure. Thus, cultures were incubated in LS containing increasing concentrations of NaCl (0, 40, 80, and 130 mM) and $[Ca^{2+}]_i$ was determined by confocal laser microscopy before and after exposure to DOM (5 μM), in the presence of MK-801 (1 μM) to prevent Ca^{2+} influx via the NMDA receptor. Extracellular concentrations of Na^+ at or below 80 mM produced a small but significant increase in Ca^{2+}-related fluorescence (from 44 to 62 fluorescence units (f.u.) localized in the cell bodies before exposure to DOM (Figure 4B), in accordance with previous studies Nijar, 1993.

Exposure of neuronal cultures to 5 μM DOM in either +NaLS or −NaLS resulted in a dramatic rise in Ca^{2+}-related fluorescence intensity in most cell bodies (Figure 4A). Domoic acid produced a rapid increase in $[Ca^{2+}]_i$, that reached a maximum value at 1 min after exposure, was independent of the extracellular Na^+ concentration and remained constant thereafter for 5 min (Figure 4B). Then, Ca^{2+}-related fluorescence began to decrease significantly in neurons incubated both in 130 and 80 mM LS and reached 60% and 80% maximum intensity, respectively, (Figs. 4A and 4B). Surprisingly, at 40 and 0 mM extracellular Na^+ concentrations, the fluorescence intensity did not decrease during the studied time frame (Figs. 4A and 4B). As shown in Figure 4B, the Ca^{2+}-related fluorescence increase induced by DOM in +NaLS (130 mM NaCl) and −NaLS (0 mM NaCl) could be totally abolished by the AMPA receptor antagonist GYKI, and no significant differences were found in fluorescence intensities (f.u.) in neurons exposed to DOM + GYKI in +NaLS (DOM + GYKI = 37 ± 1 f.u. $n = 50$) when compared with −NaLS (DOM + GYKI = 38 ± 1 f.u. $n = 60$).

The VSCaC antagonist nifedipine (NIF, 1 μM) potently reduced Ca^{2+}-related fluorescence increase induced by 5 μM DOM in +NaLS (DOM = 175 ± 4 f.u. $n = 65$; vs DOM + NIF = 62 ± 3 f.u. $n = 50$) (Figure 4C). However, NIF produced only a slight, although significant decrease in the fluorescence intensity of neurons treated with DOM in −NaLS (DOM = 173 ± 4 f.u. $n = 60$; vs NIF + DOM = 134 ± 3 f.u. $n = 60$) (Figure 4C).

Role of Ca^{2+} in Excitotoxicity by DOM in Extracellular Na^+-Free Conditions

We next tested the involvement of calcium influx in DOM-induced excitotoxicity. First we tested whether blocking VSCaCs could ameliorate neuronal survival. As shown in Table 3, NIF (1 μM) failed to reduce neurotoxicity induced by 5 μM DOM in −NaLS and did not change the morphological features of neurotoxicity 1 and 2 (swelling/darkening and neurite fragmentation, respectively, data not shown). Similarly, NIF failed to improve neuronal survival following exposure to a toxic concentration of DOM (20 μM) in physiological extracellular Na^+ concentrations as previously reported (Fernández-Sánchez and Novelli, 1996). Under physiological concentrations of sodium (130 mM), NIF (1–10 μM) promoted the excitotoxicity of an otherwise nontoxic concentration of DOM (5 μM) (% neuronal survival 8 ± 2 in +NIF vs 85 ± 12 in −NIF, DOM treated cultures), an effect already observed with subtoxic concentrations of KA (Tasker *et al.*, 2002).

We then examined the possibility that the enhancement of DOM neurotoxicity in −NaLS could be due to the activation of a proteolytic process by the sustained intracellular Ca^{2+} elevation we observed. An enzyme thought to play an important role in Ca^{2+}-mediated excitotoxic cell death is the Ca^{2+}-activated protease calpain. We examined whether the calpain inhibitor

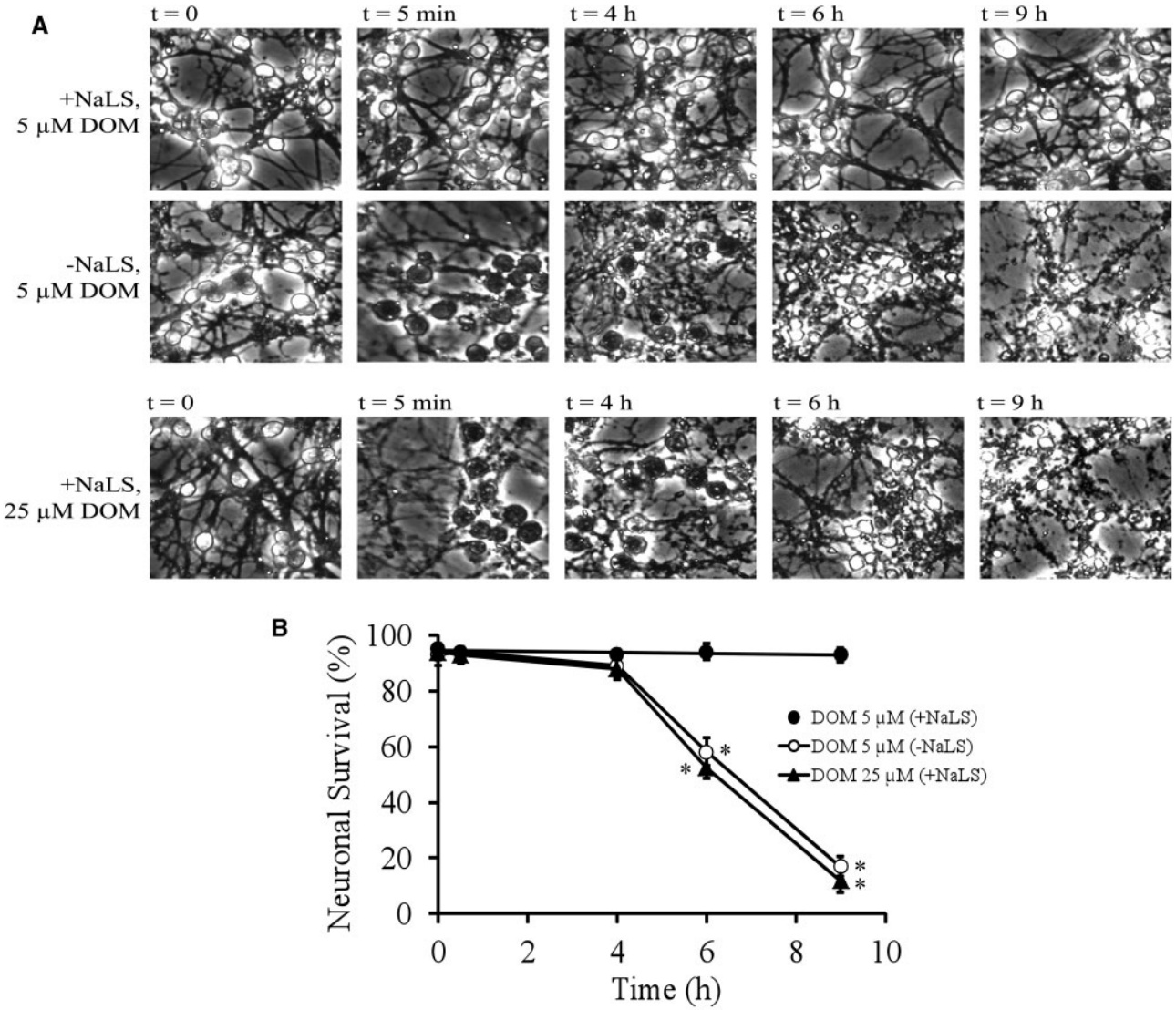

Figure 2. Chronology of DOM neurotoxicity development. A, Cultured neurons were exposed to the same concentration of DOM (5 μM) either in the presence or in the absence of extracellular Na^+, and the following morphological changes were observed by phase-contrast microscopy at the indicated times. Swelling and darkening of cell bodies after 5 min exposure to DOM in the absence of extracellular Na^+ are evident, and granulation and wakening of neuronal network were observed in the following 4 h. A higher concentration of DOM (25 μM) in the presence of extracellular Na^+ was included for further morphological comparison. B, Quantification of neuronal survival by fluorescein diacetate/ethidium bromide staining at the times indicated in (A). Data are the mean ± SD ($n = 4$ neuronal fields). *$p \leq .01$ versus DOM 5 μM +NaLS.

N-acetyl-Leu-Leu-Met-CHO (ALLM, 50 μM) could prevent DOM-induced neurodegeneration either in −NaLS or in +NaLS. As shown in Table 3, no significant differences were observed in neuronal survival to DOM following the pretreatment (1 h) with ALLM in either condition, and no changes in the morphological features of neurotoxicity were observed (data not shown).

To clarify the role of calcium in DOM excitotoxicity in our experimental conditions, neurons were incubated either in +NaLS and −NaLS in the presence of 1.5 mM EGTA to reduce the extracellular Ca^{2+} concentration to approximately 0.7 mM and then exposed to DOM (5 μM) for 1 h before replacing the incubation buffer with Basal Eagle Medium (BME). Neuronal survival was determined 24 h later. The reduction in extracellular Ca^{2+} concentration enhanced DOM-mediated excitotoxicity in neurons in +NaLS but reduced it significantly in neurons incubated in −NaLS (Table 4).

Electrophysiological Recordings

Extracellular electrophysiological recording in neurons grown on MEA devices was performed to obtain further information on the fast cellular response to DOM stimulation under our experimental conditions. Due to the absence of detectable electrical response from cerebellar neurons, we relied on cortical neurons because of their well-established responsiveness to EAAs. Cortical neuron spontaneous activity in the presence of physiological concentrations of extracellular Na^+ consisted of a random occurrence of spikes and bursts and it was either enhanced by NMDA (5 μM), AMPA (1 μM), or DOM (100–500 nM) (Supplementary Figure 1, Figure 5) or reduced by NMDA and non-NMDA receptor antagonists (Supplementary Table 1). Higher subtoxic concentrations of DOM (1–5 μM) suppressed all neuronal spontaneous activity (Figure 5, Table 5), independently of the presence of $GABA_A$ receptor antagonists (data not shown). The presence of a reduced concentration of

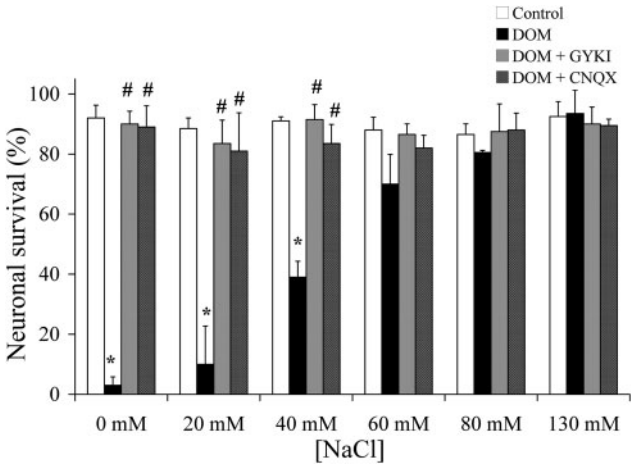

Figure 3. The enhancement of DOM neurotoxicity is inversely dependent on extracellular Na^+ concentration. Neuronal cultures were exposed to DOM (5 μM) in the presence of increasing concentration of extracellular Na^+, and neuronal survival was determined by fluorescein diacetate/ethidium bromide staining after 9 h. GYKI 52466, (GYKI, 50 μM) and (CNQX, 15 μM) were added 15 min before DOM. Data are the mean ± SD ($n = 3$ neuronal fields) *$p \leq .01$ versus DOM, 130 mM Na^+; #$p \leq .01$ versus DOM, same treatment.

Table 1. Glutamate-Mediated Excitotoxicity Is Independent of Extracellular Na^+

Treatment	Neuronal Survival (%)			
	+NaLS		−NaLS	
	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$
None	90 ± 5	85 ± 1	92 ± 3	93 ± 1
GLU 0.5 mM	87 ± 4	54 ± 13	90 ± 2	61 ± 1
GLU 1 mM	89 ± 6	13 ± 1	87 ± 5	9 ± 2

Cerebellar granule cell cultures were exposed to a GLU concentration of either 0.5 or 1 mM for 9 h either in the presence (+NaLS, 130 mM) or in its absence (−NaLS, 0 mM) of Na^+. The addition or omission of Mg2+ from the incubation buffer is indicated to evaluate the contribution of NMDA receptors on GLU-mediated excitotoxicity. Neuronal survival was quantified as described in Materials and Methods. Data are the mean ± SD ($n = 3$ neuronal fields).

extracellular Na^+ (60 mM) abolished the spontaneous activity and prevented the electrical response to DOM (Table 5).

DISCUSSION

Enhancement of DOM Neurotoxic Potency

This study shows for the first time evidence that DOM neurotoxicity is enhanced when the extracellular concentration of Na^+ is lower than 60 mM. Under the incubation conditions we used, exposure to a concentration of DOM (5 μM) that was subtoxic to cultured neurons in the presence of a physiological concentration of Na^+ (130 mM) resulted 90% of neuronal cell death in the absence of extracellular Na^+. On the other hand, in the presence of 130 mM Na^+ a concentration of at least 25 μM DOM was necessary to induce a degree of neurodegeneration comparable to that achieved in the absence of extracellular Na^+, thus indicating a 5-fold reduction in the concentration of DOM necessary to induce 90% excitotoxicity in the absence of extracellular Na^+.

Receptor Specificity

Role of non-NMDA receptors. Domoic acid neurotoxicity was completely prevented by CNQX at any extracellular Na^+ concentration, confirming the activation of ionotropic non-NMDA receptors by this excitotoxin in any condition, and a comparable neuroprotection was obtained in the presence of GYKI-52466, suggesting the activation of AMPA receptors by DOM (Figure 3). Domoic acid is an analog of KA and its excitotoxicity in cultured cerebellar neurons is compatible with a low affinity activation of AMPA receptors and the generation of a nondesensitizing response (Chittajallu *et al.*, 1999; Dingledine *et al.*, 1999). In fact, it has previously been reported that while neuronal exposure to AMPA (200 μM) did not result in a significant neurodegeneration, it reduced significantly the neurotoxicity of the subsequent exposure to DOM (20 μM) from 82% ± 3% to 37% ± 13% (Fernández-Sánchez and Novelli, 1996). Furthermore, the formation of a second messenger such as cGMP, followed a similar rule: while AMPA did not stimulate a significant cGMP increase, it reduced the cGMP increase evoked by DOM (10 μM) (Fernández-Sánchez and Novelli, 1996; Chen *et al.*, 2011).

Within the non-NMDA receptors activated by DOM, AMPA/KA receptors are mostly highly permeable to Na^+ (Chittajallu *et al.*, 1999; Dingledine *et al.*, 1999), and several studies have focused on the importance of extracellular Na^+ in excitotoxicity mediated by the activation of these receptors. Thus, earlier studies have shown that KA-mediated excitotoxicity occurs independently of extracellular Na^+ both in cerebellar granule cells (Kato *et al.*, 1991) and in Purkinje cells (Brorson *et al.*,1994). Other studies have shown that substituting NaCl for choline chloride may block AMPA-induced dark cell degeneration and it may markedly attenuate AMPA-induced edematous necrosis in Purkinje neurons (Strahlendorf *et al.*, 2001), although independent studies have shown that the removal of extracellular Na^+ prevents the electrophysiological response mediated by the activation of KA receptors without affecting the response mediated by AMPA receptors (Bowie, 2002; Paternain *et al.*, 2003; Plested *et al.*, 2008). Based on this observation, the excitotoxicity of DOM we observed in the absence of extracellular Na^+ should be independent of KA receptors and involve exclusively the activation of AMPA receptors. However, this scenario does not explain the increase in the neurotoxic potency of DOM.

Table 2. NMDA Receptors Do Not Contribute to the 3 Toxicity Features That Followed DOM Treatment Independently of the Presence of Extracellular Na^+

Treatment		Swelling and Darkening (5–30 min)		Neurite Fragmentation (6 h)		% Neuronal Survival (9 h)	
		+NaLS	−NaLS	+NaLS	−NaLS	+NaLS	−NaLS
None	−MK-801	−	−	−	−	89 ± 7	95 ± 1
	+MK-801	−	−	−	−	94 ± 1	90 ± 6
DOM	−MK-801	+	+	+	+	7 ± 2	11 ± 2
	+MK-801	+	+	+	+	6 ± 1	7 ± 3

Cerebellar granule cell cultures were exposed to DOM for 9 h in the presence of Na^+ (+NaLS, 130 mM Na^+, DOM 25 μM) or in its absence (−NaLS, 0 mM Na^+, DOM 5 μM). The addition or omission of the noncompetitive NMDA receptor antagonist MK-801 from the incubation buffer is indicated to determine the contribution of NMDA receptors to DOM-mediated excitotoxicity was added 15 min before DOM. The presence (+) or absence (−) of cell swelling and darkening at 5 and 30 min, and neurite fragmentation at 6 h were observed using a phase-contrast microscope by at least two independent authors and recorded while neuronal survival was quantified at 9 h after staining with fluorescein diacetate and ethidium bromide as described in Materials and Methods. Data are the mean ± SD ($n = 3$–4 neuronal fields).

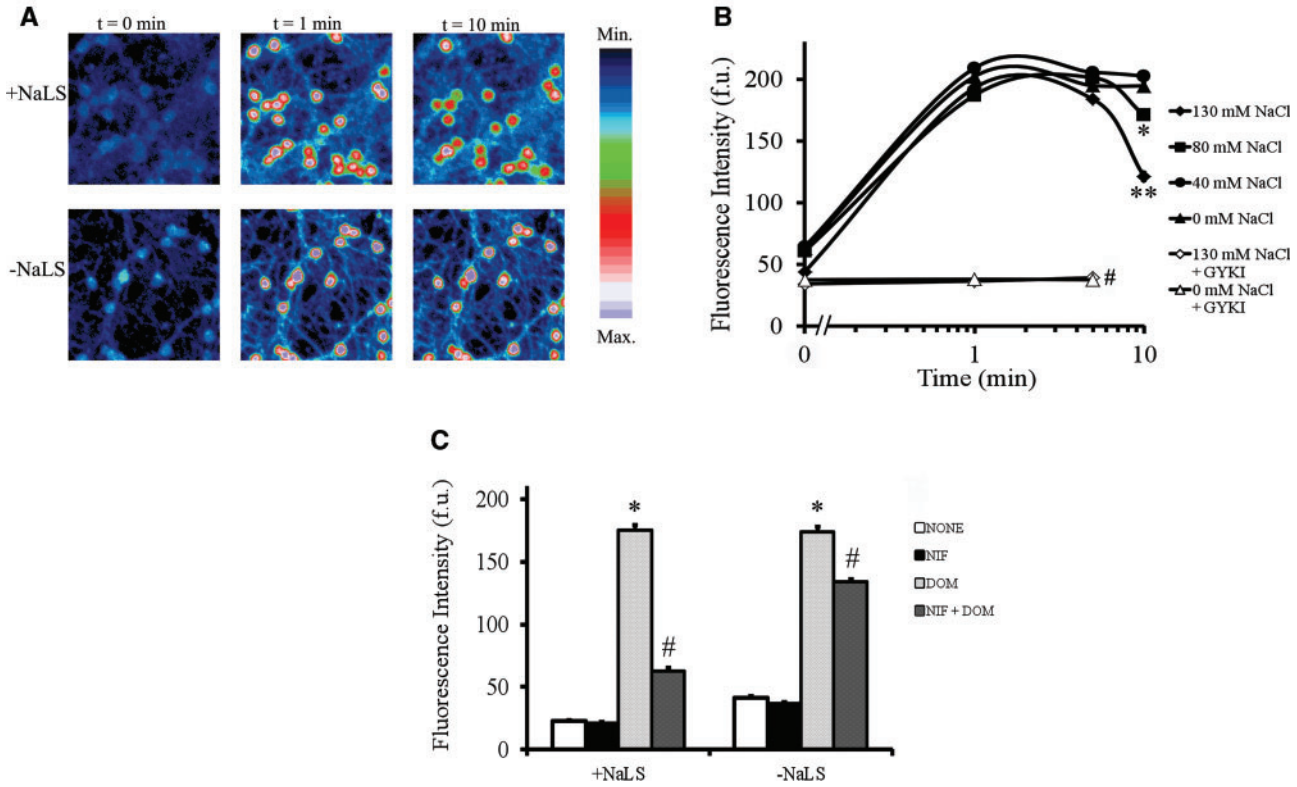

Figure 4. Kinetics of intracellular Ca^{2+} concentration following exposure to DOM are dependent on extracellular Na^+ concentrations. Neuronal cultures were incubated in a LS containing different concentrations of Na^+ and the calcium indicator Fluo-3-AM before exposure to DOM (5 μM) under a Leica confocal microscope, as described in Materials and Methods. Changes in the fluorescence emission were recorded before and after exposure to DOM during a maximum of 10 min to avoid Ca^{2+} leakage from cells due to the rapid morphological changes induced by DOM. Values represent mean ± SEM (n = 55–60 neurons). A, Intracellular Ca^{2+}-mediated fluorescence emission before and after exposure to DOM during 1 and 10 min either in the presence (+NaLS) or in the absence (−NaLS) of extracellular Na^+. B, Time course of Ca^{2+}-mediated fluorescence intensity following exposure to DOM of cultures maintained in LS-containing variable concentrations of Na^+. GYKI 52466, (GYKI, 50 μM) was added 5 min before DOM. *$p \leq .05$ and **$p \leq .01$ versus lower Na^+ concentrations; #$p \leq .01$ versus DOM at same extracellular Na^+ concentration. C, Comparison of the effect of the dihydropyridine Nifedipine (1 μM), a voltage-sensitive calcium channel blocker, on the Ca^{2+}-mediated intracellular fluorescence increase 2 min following exposure to DOM at either 130 mM Na^+ (+NaLS) or 0 mM Na^+ (−NaLS). *$p \leq .01$ versus none; #$p \leq .01$ versus DOM at same extracellular Na^+ concentration.

Role of NMDA receptors. Such increase in the neurotoxic potency of DOM appears not to involve the contribution of NMDA receptors, as the presence of the NMDA receptor antagonist MK-801 did not affect neuronal survival (Table 2). Furthermore, our data indicate the absence of enhancement of GLU toxicity mediated by the activation of NMDA receptors (Table 1), therefore reinforcing the idea that DOM toxicity enhancement in the presence of reduced extracellular concentrations of Na^+ involves a mechanism specifically related to non-NMDA receptors. We have previously shown that DOM may facilitate NMDA receptor-mediated response to GLU (Novelli *et al.*, 1992) and NMDA receptor antagonists may reduce the early phase of DOM excitotoxicity characterized by swelling and darkening of the cell body (Fernández-Sánchez and Novelli, 1996; Tasker *et al.*, 2002). A

similar result has been reported using depolarizing agents (Fernández-Sánchez *et al.*, 2001) and indicates the release of endogenous NMDA receptor agonists. Although such release may have a significant role in the extent of the final neurotoxic response (Berman and Murray, 1997; Berman *et al.*, 2002; Giordano *et al.*, 2006), our results in this study as well as in previous work (Fernández-Sánchez and Novelli, 1993, 1996; Novelli *et al.*, 1992; Tasker *et al.*, 2002), and those obtained in other experimental systems (Duran *et al.*, 1995; Larm *et al.*, 1997) provide further support to the idea that while the activation of non-NMDA receptors may cooperate in the signaling via the NMDA receptor, leading to either physiological or pathological outcomes, such as LTP for learning and memory or epilepsy, the neurodegenerative process is not necessarily based on such cooperation and may largely depend on both the choice of the experimental conditions (such as temperature and extracellular [K^+]) and the genetics/epigenetics of the primary cultures. The latter in particular may affect the affinity and the ratio of NMDA/non-NMDA receptors as well as the extent of GLU release.

Table 3. Domoic Acid Excitotoxicity Is Not Ameliorated by VSCaC Blockers or by Calpain Inhibitors

Treatment	Neuronal Survival (%)					
	None		+NIF		+ALLM	
	+NaLS	−NaLS	+NaLS	−NaLS	+NaLS	−NaLS
None	90 ± 5	93 ± 1	91 ± 1	88 ± 6	93 ± 5	94 ± 2
DOM	9 ± 2	6 ± 2	7 ± 2	7 ± 3	5 ± 1	8 ± 4

Cerebellar granule cell cultures were exposed to a toxic concentrations of DOM in the absence of extracellular Na^+ (DOM 5 μM, −NaLS) or in physiological conditions (DOM 25 μM, +NaLS). The toxicity of DOM was challenged by adding NIF (1 μM) or the calpain II inhibitor ALLM (50 μM) 15 min and 1 h, respectively, prior to the application of DOM. Neuronal survival was quantified at 9 h after the addition of DOM using the fluorescein diacetate/ethidium bromide staining as indicated in Materials and Methods. Data are the mean ± SD ($n = 4$ neuronal fields).

Table 4. Dual Role of Calcium in DOM-Mediated Excitotoxicity: Dependence on the Extracellular Na^+ Concentration

Treatment	Neuronal Survival (%)			
	None		+EGTA 1, 5 mM	
	+NaLS	−NaLS	+NaLS	−NaLS
None	90 ± 5	93 ± 1	75 ± 6	88 ± 6
DOM 5 μM	85 ± 12	21 ± 5*	14 ± 1**	64 ± 13***

Neuronal cultures were exposed to the indicated treatments for 1 h. Then, the LS was replaced with BME and neuronal survival was determined 24 h later. Data are the mean ± SD ($n = 3$ neuronal fields).
*$p < .01$ versus DOM/None/+NaLS; **$p < .01$ versus DOM/None/+NaLS; ***$p < .01$ versus DOM/None/−NaLS.

Intracellular Ca^{2+}

Our observation that in physiological conditions (+NaLS) neuronal exposure to DOM may result in higher intracellular Ca^{2+} concentrations is consistent with previous studies in a variety of neuronal preparations, including tissue slices (Nijjar, 1993), embryonic brain stem cells (König *et al.*, 1994) and primary cultures of hippocampal (Xi and Ramsdell, 1996), cortical (Beani *et al.*, 2000), and cerebellar granule cells (Fernández-Sánchez and Novelli, 1996; Savidge *et al.*, 1997; Savidge and Bristow, 1997). Non-NMDA receptors have been thought to play an indirect role based on initial studies showing that they were largely Ca^{2+}-impermeable (Mayer and Westbrook, 1987). However, several studies have demonstrated the presence of Ca^{2+}-permeable AMPA/KA receptor in specific neuronal and glial populations within the CNS, including hippocampal pyramidal cultures (Yin *et al.*, 1999), cortical neurons (Lu *et al.*, 1996), and cerebellar granule cells (Savidge *et al.*, 1997; Savidge and Bristow, 1997). The cloning of a number of non-NMDA receptors subunits has indicated the molecular basis for their permeability or impermeability to Ca^{2+}. A single amino acid change in GluR2 receptor subunit, resulting from transcriptional editing of the mRNA, renders the resulting AMPA/KA receptor Ca^{2+} impermeable; unedited versions of the subunits produce Ca^{2+} permeable receptors (for review see Novelli *et al.*, 2005), and a mosaic of both Ca^{2+} permeable and Ca^{2+} impermeable receptors can be coexpressed in the same cells when edited GluR2 is expressed with GluR4 or when editing of the GluR2 mRNA is partial (Burnashev *et al.*, 1992). Within this complex diversity, our results showing that the [Ca^{2+}]i increase we observed following neuronal exposure to DOM in the presence of physiological Na^+ concentrations was fully prevented by non-NMDA receptor antagonists and largely prevented by dihydroperidines, support the idea that most of the calcium enters the neurons via the L-type VSCaC (Murphy and Miller, 1989) that becomes activated following the depolarization produced by the influx of Na^+ via non-NMDA receptors activated by DOM (Fernández-Sánchez and Novelli, 1996). Such influx of calcium is functional to the

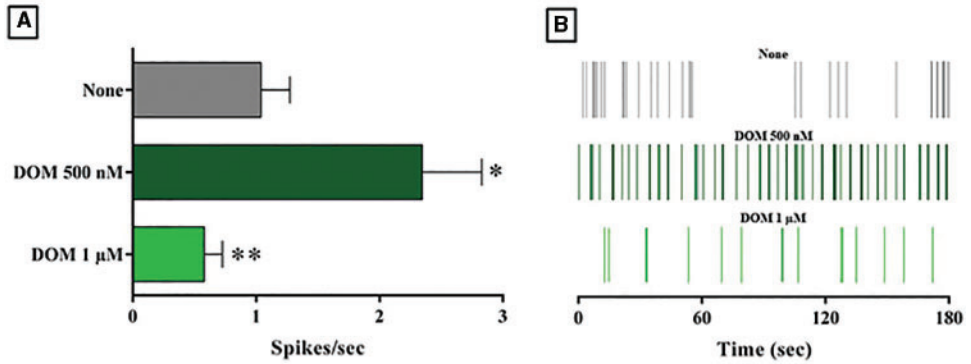

Figure 5. Concentration-dependent transition from stimulatory to inhibitory effect of DOM on neuronal electrical activity. A, Concentrations of DOM up to 500 nM increased the n° of spikes/s in neuronal electrical activity, while concentrations ≥ 1 μM DOM depressed spontaneous neuronal activity. Spikes/s values are reported in Table 5. *$p \leq .05$ versus none; **$p \leq .01$ versus DOM 500 nM. B, Recordings of whole neuronal activity in the absence and in the presence of either 500 nM or 1 μM DOM.

Table 5. Concentration-Dependent Transition from Stimulatory to Inhibitory Effect of DOM on Neuronal Electrical Activity

	130 mM Na^+		60 mM Na^+	
	Spikes/s	Bursts/min	Spikes/s	Bursts/min
None	1.04 ± 0.22 (100%)	6.81±1.59 (100%)	n.d.	n.d.
DOM 0.5 μM	2.35 ± 0.46 (226%)	10.04 ±1.23 (147%)	n.d.	n.d.
DOM 1 μM	0.58 ± 0.14 (56%)	3.96 ± 0.41 (58%)	—	—
DOM 2 μM	n.d.	n.d.	—	—

The electrical activity of cultured cortical neurons grown on multi electrode array s (MEAs) was measured in the presence of either 130 or 60 mM extracellular Na^+ in the incubation buffer. Two main parameters are reported: spikes/s and burst/min. Data obtained in the presence of 130 mM Na^+ are from Figure 5. In the presence of 60 mM extracellular Na^+, no electrical activity could be recorded neither in spontaneous conditions nor after stimulation by DOM. n.d., not detectable. Values are the mean ± SEM ($n = 10–20$ electrodes).

activation of cGMP synthesis that increases progressively following neuronal exposure to DOM concentrations > 1 μM (Chen *et al.*, 2011). However, assuming a similar sensitivity to EAAs in cortical and cerebellar neurons where the electrical activity could not be monitored by MEA, the stimulation of cGMP synthesis may not parallel the increase of spontaneous activity produced by concentrations of DOM within the 100–500 nM range. Interestingly, neuronal exposure to higher concentrations of DOM (1–5 μM), capable of both, increasing the intracellular Ca^{2+} concentration and initiating a Ca^{2+}-dependent cGMP formation without toxic effects (Chen *et al.*, 2011), depressed neuronal electrical activity as reported in similar studies (Hogberg *et al.*, 2011; Mack *et al.*, 2014; Wallace *et al.*, 2015). It is worth noting that the inhibition of neuronal electrical activity we observed with DOM was not prevented by the presence of $GABA_A$ receptor antagonists, ruling out an inhibitory effect mediated by the release of GABA and suggesting the occurrence of a stimulation on the limits of the neuronal capability to generate the action potentials recorded by the MEA system. The scenario of DOM action in the presence of lower than physiological Na^+ concentrations can be more complicated, as indicated by the observed absence of spontaneous neuronal activity in our extracellular recordings when extracellular Na^+ concentration is ≤ 60 mM. Although this observation is not surprising given that action potentials are recorded extracellularly, this result underscores the fact that the depolarization that activates the dihidroperidine-sensitive VSCaCs, still contributing to the Ca^{2+} response to DOM in −NaLS conditions, cannot be due to Na^+ ions. Thus, considering that KA receptors are inactive in these conditions (Bowie, 2002; Paternain *et al.*, 2003; Plested *et al.*, 2008), Ca^{2+} ions entering the edited AMPA receptors may likely have a depolarizing effect by accumulating in the cytoplasm due to the impaired functioning of the Na^+/Ca^{2+} exchanger (Berman and Murray, 1997; Berman *et al.*, 2002; Kiedrowski *et al.*, 1994; Kiedrowski and Costa, 1995). Accordingly, in +NaLS after 10 min-course of the experiment, Fluo-3 fluorescence was significantly reduced, but in −NaLS, the fluorescence intensity did not decrease.

Calcium permeability of the AMPA receptor. The Ca^{2+} permeability of the edited population of AMPA receptors is particularly evident in the absence of extracellular Na^+, when comparing the peak intracellular Ca^{2+} increase in the absence and in the presence of the VSCaC blocker NIF. The absence of extracellular Na^+ influx following the activation of AMPA receptors by DOM guarantees a triple effect: (1) a higher electrical driving gradient to the Ca^{2+} influx; (2) a higher driving chemical Ca^{2+} gradient by nullifying the early Ca^{2+} influx from VSCaCs; and (3) the inactivation of the Na^+/Ca^{2+} exchanger. It is worth noting that DOM is key in demonstrating Ca^{2+} permeability because of its block of AMPA receptor desensitization (Novelli *et al.*, 2014; Savidge and Bristow, 1997). It has been proved that excessive entry of Ca^{2+} into granule cells induced a fast collapse of the mitochondrial membrane potential (Ward *et al.*, 2000). Within a cell, balance between Na^+ and Ca^{2+} ions could determine the probability of cell damage. In our preliminary experiments, we did not observe a significant improvement of cell survival using the calpain inhibitor ALLM, although additional experiments would be required to clarify calpain implication in a neurodegenerative process participated by mitochondrial dysfunction.

Our results indicate that calcium may have a dual role in the excitotoxic process initiated by DOM: it appears to counteract it in physiological conditions and to promote it when an ionic imbalance is present. In the former condition, neuronal protection is mediated by VSCaC being responsible for most of the calcium influx while in the latter condition their role is not significant with respect to the overall calcium influx. Thus, we speculate that the neuronal scaffolding hosting VSCaC may provide the proper use for calcium influx toward neuroprotection and trophism, while calcium influx via other structures may represent a potential problem for neuronal survival.

Relevance to Hyponatremia

It is well-known that the occurrence of lower than physiological concentrations of Na^+ in the blood, hyponatremia, is a relatively frequent finding in diabetics and in elderly people and may become a potentially life-threatening condition if left untreated (Hoorn and Zietse, 2008; Gunathilake *et al.*, 2013). Although none of the elderly patients that developed cognitive dysfunction during the Canadian episode of DOM intoxication in 1987 (Perl *et al.*, 1990; Teitelbaum *et al.*, 1990) was reported to be hyponatremic, the occurrence of such a coincidence cannot be excluded in the general population and particularly in the elderly consuming seafood contaminated with DOM. In fact, several neuroactive drugs, such as valproic acid and antidepressants, are known to cause severe hyponatremia via mechanisms that are not fully understood, with the elderly being the most affected group (Mannesse *et al.*, 2013; De Picker *et al.*, 2014; Gupta *et al.*, 2015; Leth-Møller *et al.*, 2016).

Our results show that the potentiation of DOM excitotoxicity occurs at extracellular Na^+ concentrations below 80 mM, lower than that reported in one of the most severe reported case of hyponatremia where the serum sodium concentration was as low as 99 mM (Gupta *et al.*, 2015). However, it is possible that drug-induced serum hyponatremia may cause a more severe reduction of extracellular Na^+ at the synaptic level. In a recent review, Agnati *et al.* (2017) suggested the existence of local homeostatic differences in the brain interstitial fluid surrounding the neurons, particularly with respect to the concentration of Na^+, finally resulting in lower concentrations in the CSF compared with serum during the circadian rhythm. Thus, it cannot be excluded that drug-induced hyponatremia may eventually become severe enough in synaptic surroundings to facilitate DOM excitotoxicity. Although this hypothesis may be worth further studies, we believe that our results may be relevant to set new prudential safety limits of DOM levels in seafood.

CONCLUSIONS

In conclusion, our results provide novel evidence indicating a main role of extracellular Na^+ concentration in the physiological control of the response to DOM in cerebellar granule cells. In particular, this study points out the importance of physiological extracellular Na^+ concentrations for a proper control of intracellular Ca^{2+} concentrations changes following the activation of AMPA receptors by this marine toxin, making the difference between a physiological and a toxicological outcome.

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SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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SUPPLEMENTARY DATA:

SELECTIVE ENHANCEMENT OF DOMOIC ACID TOXICITY IN PRIMARY CULTURES OF CEREBELLAR GRANULE CELLS BY LOWERING EXTRACELLULAR Na^+ CONCENTRATION

Table 1S.

Changes in spontaneous electrical activity following treatment with excitatory amino acid receptor antagonists

Changes in spontaneous activity vs untreated (%)		
	Total Spikes/sec	Total Bursts/min
CNQX (15μM)	-67±22	-73±15
MK-801 (1μM)	-83±18	-87±20
Mg^{2+} (1mM)	-98± 2	-98± 2

The spontaneous electrical activity of cultured cortical neurons grown on microelectrode arrays (MEAs) was measured in the presence of several ionotropic glutamate receptor antagonists in the incubation buffer and compared with that in their absence. Two main parameters are reported: spikes/sec and burst/min. Values are the mean ± SD (n=10-20 electrodes).

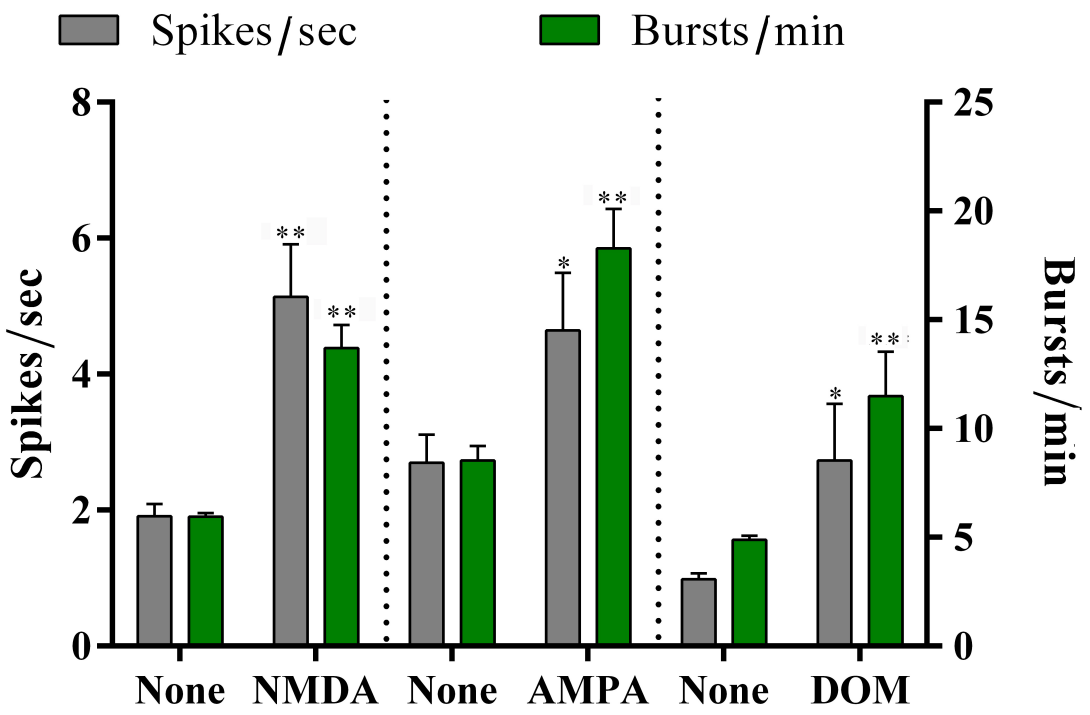

Fig. 1S Extracellular recordings of spontaneous and glutamate analogues-induced electrical activity in cultured neurons.

Cortical neurons were cultured on a microelectrode array (MEA) allowing for extracellular recording of their electrical activity, here reported for two major parameters: spikes/sec and burst/min. The response to glutamate receptor agonists (NMDA, 5μM; AMPA, 1μM; DOM 100 nM) was recorded 10 min after their addition to the culture, and lasted several hours. Value are the mean ± SEM (n=10-20 electrodes) * P≤0.05; ** P≤ 0.01