# CHRONIC CIGUATOXIN TREATMENT INDUCES SYNAPTIC SCALING THROUGH VOLTAGE GATED SODIUM CHANNELS IN CORTICAL NEURONS

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**Key words: CTX 3C, Arc, Egr1, voltage gated sodium channels, cortical neurons, synaptic scaling, mEPSCs, glutamate receptors.**

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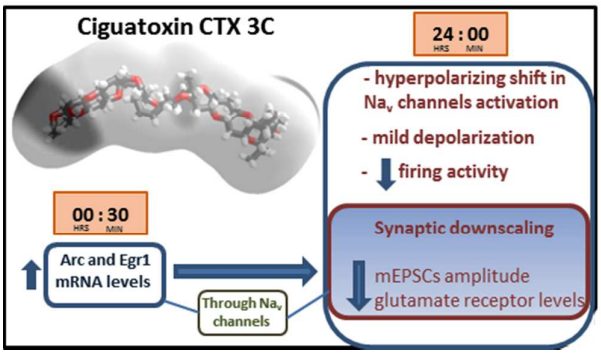

## ABSTRACT

Ciguatoxins are sodium channels activators that cause ciguatera, one of the most widespread nonbacterial forms of food poisoning, which presents with long term neurological alterations. In central neurons, chronic perturbations in activity induce homeostatic synaptic mechanisms that adjust the strength of excitatory synapses and modulate glutamate receptors expression in order to stabilize the overall activity. Immediate early genes, such as Arc and Egr1, are induced in response to activity changes and underlie the trafficking of glutamate receptors during neuronal homeostasis. To better understand the long lasting neurological consequences of ciguatera, it is important to establish the role that chronic changes in activity produced by ciguatoxins represent to central neurons. Here, the effect of a 30 min exposure of 10-13 days *in vitro* (DIV) cortical neurons to the synthetic ciguatoxin CTX 3C on Arc and Egr1 expression was evaluated by using real time polymerase chain reaction approaches. Since the toxin increased the mRNA levels of both Arc and Egr1, the effect of CTX 3C in Nav channels, membrane potential, firing activity, miniature excitatory postsynaptic currents and glutamate receptors expression in cortical neurons after a 24 hours exposure was evaluated by using electrophysiological and western blot approaches. The data presented here show that CTX 3C induced an upregulation of Arc and Egr1 that was prevented by previous coincubation of the neurons with the $Na_v$ channel blocker tetrodotoxin. In addition, chronic CTX 3C caused a concentration-dependent shift in the activation voltage of $Na_v$ channels to more negative potentials and produced membrane potential depolarization. Moreover, 24 hours treatment of cortical neurons with 5 nM CTX 3C decreased neuronal firing and induced synaptic scaling mechanisms as evidenced by a decrease in mEPSCs amplitude and downregulation in the protein levels of glutamate receptors that was also prevented by tetrodotoxin. These findings identify an unanticipated role for ciguatoxin in the regulation of homeostatic plasticity in central neurons involving $Na_v$ channels and raise the possibility that some of the neurological symptoms of ciguatera might be explained by these compensatory mechanisms.

## INTRODUCTION

Ciguatera fish poisoning is a seafood-borne illness caused by the consumption of contaminated fish that have accumulated toxins produced by marine dinoflagellates of the genus *Gambierdiscus*. This food poisoning is endemic in several tropical and subtropical areas, however, its occurrence is increasing worldwide and several cases have recently appeared in Europe.[1,2]

Ciguatoxin (CTX), the most prevalent toxin in ciguatera, has a long semirigid ladder-shaped polyether structure with fused ether rings of different size and its main mechanism of action consists in activating $Na_v$ channels producing cell membrane depolarization at rest.[3-7] In humans, ciguatera fish poisoning presents with neurological symptoms, that may last for several months or even years, including paraesthesia, headache, weakness, heightened nociperception and sensory abnormalities such as pruritus, arthralgia, myalgia, and dental pain.[8,9] However, the underlying cellular alterations for these symptoms are not understood so far. It has been reported that the high lipid solubility of CTX and its long retention in neuronal membranes can account for the chronic nature of these neurological sequels,[8] which, traditionally, have been related to the direct interaction of CTX with Nav channels.[10,11] However, there is a lack of information about the *in vitro* effects of a chronic exposure of neurons to CTX. In this sense, shifts in intracellular sodium concentration are involved in several forms of synaptic plasticity that depend on neuronal activity[12-15] and thus, we here hypothesized that some of the neurological perturbances of ciguatera could be related to alterations in synaptic transmission induced by CTX 3C.

We recently described that an acute administration of 5 nM CTX 3C to cortical neurons shifts the current-voltage curve of $Na_v$ channels activation in the hyperpolarizing direction without affecting $K_v$ channels, voltage gated calcium channels or calcium influx.[16] Moreover, acute treatment of cortical neurons with CTX 3C caused a large membrane depolarization, that was prevented when tetrodotoxin was previously added to the bath solution, and was accompanied by a suppression in firing activity, a decrease in excitatory synaptic transmission and an increase in inhibitory synaptic transmission (companion paper). However, in that study it remained unclear whether neuronal activity was reduced by CTX 3C or, in contrast, if it was activated but this activation was masked (suppression of firing activity) due to the strong depolarization

produced by the toxin. Since a depolarizing stimulus should enhance firing activity, spike counting is a potentially ambiguous measure because low levels of depolarization (that are below threshold for spike generation) and saturating levels of depolarization (that inactivate sodium channels), both result in the abolishment of action potential generation.[17]

Other indicators of changes in neuronal activity are immediate early genes (IEGs). These are the first group of genes expressed following synaptic activation and encode a diverse range of proteins including regulatory transcription factors, structural proteins, signal transduction proteins, growth factors, and enzymes[18] and can be divided into direct effector IEGs and regulatory transcription factors IEGs (RTF IEG). Direct effector IEGs, such as Arc (activity regulated cytoskeleton-associated protein), act directly to modulate specific cellular functions (synaptic structure and function), whereas RTF IEGs like Egr1 (early growth response protein 1), influence cellular physiology by increasing expression of specific downstream genes.[19] Both Egr1 and Arc are increased by heightened neuronal activity and are implicated in homeostatic synaptic mechanisms.[20-22]

Homeostatic mechanisms play an essential role in the formation, maintenance and modification of neuronal circuits, allowing neurons to adapt to changes in activity.[23-25] One of the best-characterized forms of neuronal adaptation to activity perturbations is the homeostatic response occurring at excitatory synapses of the mammalian central nervous system, a process called synaptic scaling.[26] By this mechanism neurons upregulate or downregulate the amplitude of miniature excitatory postsynaptic currents (mEPSCs), in an activity-dependent manner and in the direction needed to maintain stability.[27] mEPSCs are a valuable tool to measure changes in synaptic strength since they represent the postsynaptic response to the release of individual neurotransmitter vesicles.[26] While changes in the frequency of mEPSCs indicate actions on presynaptic neurotransmitter release,[28] changes in the amplitude of mEPSCs are indicative of changes in the number of postsynaptic AMPA and NMDA glutamate receptors.[26,27,29,30] However, homeostatic synaptic mechanisms, such as synaptic scaling, have a slow time course and require hours to days of altered activity to produce changes in synaptic strength and to scale up or down proportionally all excitatory synapses.[26]

The main objective of this work was to evaluate whether CTX 3C produced an elevation of network activity through $Na_v$ channels and to analyze the effect of a chronic exposure of 10-13 DIV cortical neurons to CTX 3C. A 30 min exposure of cortical neurons to CTX 3C induced a concentration-dependent upregulation of the IEGs Arc and Egr1, which was prevented by the $Na_v$ channels antagonist tetrodotoxin. After 24 h of treatment, 5 nM CTX 3C shifted sodium channel activation to more negative voltages, produced membrane potential depolarization, decreased neuronal firing activity, increased the frequency but decreased the amplitude of mEPSCs and reduced the expression of NMDA and AMPA receptor subunits which was also prevented by previous addition of tetrodotoxin. Overall, these results indicate that CTX 3C induced synaptic scaling in cortical neurons and this effect involved a rapid increase in the mRNA levels of activity-dependent IEGs as well as an indirect modulation of glutamate receptors expression in a $Na_v$ channels dependent manner. The data presented here provide new insights to understand the neurological effects observed in animal models and humans after ciguatera food poisoning.

## MATERIAL AND METHODS

### Primary cultures of cortical neurons

Swiss mice were used to obtain primary cultures of cortical neurons. All protocols were approved by the University of Santiago de Compostela Institutional animal care and use committee. Primary cortical neurons were obtained from embryonic day 16–18 Swiss mice as previously described.[16] All data were obtained in parallel from drug-treated and age-matched sister control cultures treated with DMSO 0.1% as a vehicle.

### RNA purification and real time PCR

RNA from control and treated cells was purified using the Aurum™ Total RNA Mini Kit (Bio-Rad) following the manufacturer's instructions. RNA concentration was determined with a NanoDrop 2000 (Fisher Scientific). Equal amounts of total RNA from each sample were reverse transcribed using an oligo-dT (Fisher Scientific) and a RevertAid™ M-MuLV reverse transcriptase (Fisher Scientific) following the manufacturer's instructions. The Arc, Egr1 and RPL13A (housekeeper) cDNAs were amplified in a StepOne real time PCR (Applied Biosystems) using the iTaq™ Universal SYBR(R) Green Supermix (Bio-Rad),

following the manufacturer's instructions (Table 1 shows primers and targets sequences). Results were analyzed using the StepOne software (Applied Biosystems) and relative quantification between treatments was performed using the ΔΔCt method[31]. Each treatment was performed in triplicate.

**Electrophysiology**

Whole cell patch-clamp recordings, achieved by gentle mechanical suction of the membrane patch, were performed on cortical neurons between 10-13 days in culture, at room temperature (22-25ºC). A computer-controlled current and voltage clamp amplifier (Multiclamp 700B, Molecular Devices) was used. Signals were recorded and analyzed using a Pentium computer equipped with Digidata 1440 data acquisition system and pClamp10 software (Molecular Devices, Sunnyvale, CA). pClamp10 was used to generate current and voltage-clamp commands and to record the resulting data. Signals were filtered at 10 kHz and digitized at 20 μs intervals. Culture medium was exchanged with several washes of recording solution immediately prior to the experiment. After establishing the whole-cell configuration, neurons were allowed to stabilize for at least 5 min, before experiments were initiated, to ensure adequate equilibration between the internal pipette solution and the cell interior. Recording electrodes were fabricated from borosilicate glass micro capillaries (outer diameter 1.5 mm), and the tip resistance was 5-10 MΩ. Only recordings with stable access resistance and stable holding current for at least 3 min were included in the analysis. The external solution in all the experiments contained (in mM): 119 NaCl, 5.9 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 1.2 $NaH_2PO_4$, 22.8 $NaHCO_3$ and 0.1% glucose. (pH 7.4 adjusted with $CO_2$ prior to use). The intracellular pipette solutions contained (in mM): 150 KCl, 2 $MgCl_2$, 5 HEPES, 1.1 EGTA and 2 $Na_2ATP$ (pH 7.2). In current clamp experiments a threshold of 8 mV was established for considering neuronal spikes. Spontaneous postsynaptic currents were recorded in voltage-clamp mode while holding the postsynaptic neuron at -80 mV. Events were counted and analyzed for a 2 min period per cell in each condition. Miniature excitatory postsynaptic currents (mEPSCs) were recorded in the presence of the $GABA_A$ receptor antagonist bicuculline (BIC) at 20 μM, and the $Na_v$ channel blocker tetrodotoxin (TTX) at 0.5 μM, added to the bath solution 10 minutes prior to the experiments.[27] mEPSCs were confirmed by their complete inhibition in the presence of 20 μM 6-cyano-7-nitroquinoxaline-2,3-

dione (CNQX) and 100 μM D(-)-2-amino-5-phosphonopentanoic acid (APV). Both miniature and spontaneous postsynaptic currents were detected using an automatic template detection program (pCLAMP; Molecular Devices) and verified manually. To generate the average trace of mEPSC for a given experimental condition, all the events from every neuron recorded for two minutes per condition were averaged.[27] For exposure of cortical neurons to ciguatoxin, 24 h immediately before use half of the dishes in a set of cultures were treated with CTX 3C alone or in the presence of TTX, whereas the other half were treated with vehicle (DMSO). All data were obtained in parallel from CTX 3C-treated and age-matched sister control cultures.

**Western Blotting**

After 24 hours of treatment with either TTX, CTX 3C or a co-incubation of both compounds, 10 days *in vitro* neurons were lysed in 50 mM Tris-HCl buffer (pH 7.4) containing a phosphatase/protease cocktail inhibitor (Roche). The protein concentration in the lysates was determined by the Bradford assay. Samples of cell lysates containing 20 μg of total protein were resolved in gel loading buffer (50 mM Tris-HCl, 100 mM dithiotreitol, 2% SDS, 20% glycerol, 0.05% bromophenol blue, pH 6.8) by SDS−PAGE and transferred onto PVDF membranes (Millipore). The Snap i.d. protein detection system was used for blocking and antibody incubation as previously described.[32] The concentrations of primary antibodies employed in this work are shown in Table 1. The immunoreactive bands were detected using the Supersignal West Pico chemiluminescent substrate (Pierce) and the Diversity 4 gel documentation and analysis system (Syngene, Cambridge, U.K.). Chemiluminescence was measured with the Diversity GeneSnap software (Syngene). β-Actin was used as control for lane loading and to normalize chemiluminescence values. Each condition was analyzed per duplicate in each experiment and at least 4 experiments were performed per antibody.

**Toxins and drugs used**

The standard of CTX 3C was synthesized by Dr. Masahiro Hirama following previosly described procedures[33-35] and dissolved at a concentration of 10 μM in DMSO. Following dilutions were performed in deonized water. TTX was purchased from CIFGA (Lugo, Spain), APV and BIC were purchased from Sigma and CNQX was from Tocris. The final concentration of compound

solvent (DMSO) was less than 0.01%. All other chemicals were reagent grade and purchased from Sigma.

**Statistical Analysis**

Data are expressed as means ± standard error of the mean (s.e.m) of n determination. Error bars on the graphs represent s.e.m. Statistical comparisons between two groups were made by two tailed paired Student's t test. p values < 0.05 were considered statistically significant.

## RESULTS

**CTX 3C enhances the expression of Arc and Egr1 through $Na_v$ channels**

The first genes that undergo modulation of expression after neuronal stimulation are immediate early genes such as Arc and Egr1, whose induction occur within minutes and is transient in nature.[36-38] Since the induction of Arc mRNA peaks within 30 min and 2 hours of elevated network activity,[38] the effect of CTX 3C on Arc and Egr1 mRNA expression was measured after 30 min of exposure of cortical neurons to the toxin. The effect of CTX 3C on the mRNA levels of Arc and Egr1 is shown in Figure 1. In cortical neurons, 30 minutes exposure to 5 nM CTX 3C produced a fivefold increase in Arc mRNA levels and a threefold increase in Egr1 mRNA levels compared to control conditions and the effect of the toxin on IEG expression was completely reverted by preincubation of the neurons with 0.5 μM tetrodotoxin before the 30 min application of CTX 3C, as illustrated in Figure 1A. However, when cells were incubated for 10 minutes with TTX after the 30 min CTX 3C treatment (Figure 1B), the increase in Arc and Egr1 mRNA levels induced by CTX 3C was not affected by sodium channel blockade indicating that the effect of this toxin on the levels of these IEGs are primarily mediated by activation of TTX-sensitive $Na_v$ channels. In order to establish the concentration-dependence for the effects of CTX 3C on gene expression, further experiments were performed treating cortical neurons with CTX 3C at concentrations of 0.5 nM and 2 nM. As shown in Supplementary Figure 1, at 0.5 nM the toxin increased the mRNA levels of Arc but did not affect the mRNA levels of Egr1, although at 2 nM the toxin largely increased the levels of both genes after 30 minutes of treatment.

**Effect of chronic CTX 3C treatment on voltage gated potassium and sodium channel currents**

Once confirmed that CTX 3C produces activation of cortical networks, the effect of a chronic exposure of cortical neurons to the toxin was evaluated. First, the effect of a 24 h exposure of cortical neurons to CTX 3C was evaluated in $Na_v$ and $K_v$ channels since the primary target of CTXs are $Na_v$ channels but CTXs can also act on $K_v$ channels in some neuronal preparations.[39] However, the effect of a chronic exposure of cortical neurons to CTXs on these channels has not been previously evaluated. In all experiments neurons were treated with the synthetic ciguatoxin CTX 3C at 5 nM, a concentration that causes a rapid activation of $Na_v$ channels after a 5 minutes exposure of cortical neurons and produces a rapid membrane depolarization (companion paper) but is not neurotoxic for cortical neurons at 24 hours.[16] To measure voltage gated $K^+$ currents ($I_K$), neurons were voltage clamped at a membrane holding potential of -60 mV, and total $I_K$ was evoked by 200 ms depolarizing pulses from $V_m$ to +75 mV in 15 mV steps (Figure 2, inset). Voltage gated potassium currents were not studied in isolation during the course of these experiments, but the effect of CTX 3C on voltage-gated sodium currents ($I_{Na}$) was evaluated in parallel (see materials and methods). Thus, whole-cell currents consisted of voltage-gated sodium currents (downward deflection in the current recording) and voltage-gated potassium currents. Representative potassium and sodium currents in control cells and in neurons treated with 5 nM CTX 3C for 24 h are shown in Figure 2A and Figure 2B, respectively. As illustrated in Figure 2C, the current-voltage curve of $K_V$ was not affected by the toxin (n = 10 control cells and n = 17 cells treated with 5 nM CTX 3C for 24 hours) neither it was the $I_K$ activation threshold (about -55 mV). However, as shown in the left panel of Figure 2D, in treated neurons $I_{Na}$ was decreased by 62.9 ± 5.8 % ($p < 0.001$; n = 10 control cells and n = 17 treated cells). Moreover, CTX 3C shifted the $I_{Na}$ activation threshold to more negative membrane potentials. As shown in the right panel of Figure 2D, the $I_{Na}$ activation threshold was shifted from -36.2 ± 3.9 mV in control cells to -54.4 ± 5.6 mV in CTX 3C treated cells ($p = 0.002$; n = 12 control cells and n = 11 cells treated for 24 h with 5 nM CTX 3C).

**Chronic CTX 3C exposure induces depolarization and decreases neuronal spiking in cortical neurons**

Ciguatoxins cause membrane depolarization in several cellular models.[40] In order to evaluate the effect of 24 h exposure of cortical neurons to CTX 3C on membrane potential and spiking activity, whole-cell current clamp experiments were performed in 10-13 DIV cortical neurons. In view of the effect of 0.5 nM and 2 nM CTX 3C on mRNA levels, neurons were treated for 24 hours with those toxin concentrations (Supplementary Figure 2). As illustrated in Supplementary Figure 2A (left and right panels) at these concentrations the toxin caused a small decrease in the frequency and the amplitude of neuronal spikes although this decrease did not reach statistically significant difference versus control neurons. Moreover, as shown in Supplementary Figure 2B, left panel, treatment of cortical neurons with 0.5 nM CTX 3C for 24 hours did not affect the membrane potential. In contrast, at 2 nM the toxin depolarized the membrane potential of cortical neurons from -49.8 ± 3.8 mV in control cells to -36.6 ± 3.3 mV in neurons treated with 2 nM CTX 3C ($p = 0.02$) and this effect was completely reversed when 0.5 µM TTX was added to the bath. In view of the different sensitivity of neuronal spiking and membrane potential to 2 nM CTX 3C, the toxin was also evaluated at a concentration of 5 nM.

As shown in Figure 3, the firing activity of cortical neurons was reduced after 24 hours treatment with 5 nM CTX 3C. A typical spiking pattern of a control neuron and a neuron treated with 5 nM CTX 3C for 24 h and the resting membrane potential in both cases are shown in Figure 3A (upper and lower panel, respectively). As shown in Figure 3B (left panel), the firing rate was 1.0 ± 0.2 Hz in control conditions whereas in neurons treated with 5 nM CTX 3C, this rate was reduced to 0.5 ± 0.1 Hz ($n = 20$ and $n = 19$ respectively; $p = 0.03$). Moreover, as indicated in the middle panel of Figure 3B the amplitude of the spikes was reduced from 16.4 ± 1.8 mV in control conditions to 8.6 ± 1.6 mV after 24 hours exposure to 5 nM CTX 3C ($n = 20$ and $n= 19$ respectively; $p = 0.002$) and the resting membrane potential was significantly depolarized from -54.4 ± 1.0 mV in control to -42.5 ± 2.1 mV after treatment with 5 nM CTX 3C ($p < 0.001$, Figure 3B, right panel). Therefore, the following experiments intended to evaluate the effect of the toxin on synaptic scaling using a toxin concentration of 5 nM.

**Chronic CTX 3C did not alter mixed spontaneous postsynaptic currents in cortical neurons**

Spontaneous postsynaptic currents, sPSCs, result from a spontaneous action potential in a presynaptic neuron[41] and are involved in the regulation of postsynaptic firing and synapse homeostasis.[42,43] A typical recording of mixed sPSCs of a control neuron and a neuron treated with 5 nM CTX 3C for 24 h is shown in Figure 4A upper and lower panel, respectively. As shown in Figure 4B, the amplitude, the frequency and the area of mixed sPSCs were not affected in treated neurons (n = 25) when compared to control neurons (n = 31).

**Chronic CTX 3C, at 5 nM, increases the frequency but decreases the amplitude of miniature excitatory postsynaptic currents**

Neurons maintain stable firing rates through homeostatic regulation of many aspects of neuronal excitability such as the regulation of excitatory synaptic strength.[44] The enhancement in the levels of Arc and Egr1 mRNA by CTX 3C were indicative of an increase in synaptic activity. These IEGs are implicated in the molecular mechanisms of homeostatic plasticity[20,38] and it has been reported that Arc mediates the homeostatic response to increased activity by a pathway that ultimately downscales excitatory synaptic strength.[45] For the purpose of evaluating the effect of CTX 3C on mEPSCs, these events (isolated as described in the materials and methods section) were analyzed in control neurons and in neurons treated with 5 nM CTX 3C (Figure 5). Representative recordings of mEPSCs from control neurons (upper panel) and neurons exposed to 5 nM CTX 3C for 24 h (lower panel) are shown in Figure 5A. As shown in the left panel of Figure 5B, neurons exposed for 24 hours to CTX 3C exhibited an increase in the frequency of mEPSCs from 0.7 ± 0.2 Hz in control conditions to 2.1 ± 0.6 Hz in treated neurons ( n = 6 and n= 7 respectively, $p < 0.05$). However, as shown in the right panel of Figure 5B, 5 nM CTX 3C significantly decreased mEPSCs amplitude from 33.8 ± 4.0 pA in control conditions to 24.8 ± 2.6 pA in treated neurons (n = 6 and n= 7 respectively, $p < 0.05$). Average mEPSCs of control neurons and CTX 3C-treated neurons are shown in the left panel of Figure 5C. mEPSC kinetics were examined by scaling and overlaying average mEPSCs waveforms. This revealed no differences in

the rise time kinetics, however, the decay time was slightly slower in treated neurons (Figure 5C right panel). The effects of 0.5 nM and 2 nM CTX 3C on the frequency and amplitude of mEPSCs were also analyzed but not statistically significant differences were found (Supplementary Figure 3). Representative traces of mEPSCs recorded in control neurons (upper panel) and from the same batches of cultures treated for 24 h with 0.5 nM CTX 3C (middle panel) or 2 nM CTX 3C (lower panel) are shown in Supplementary Figure 3A. As illustrated in Supplementary Figure 3B, the pooled results for the effect of a 24 hours exposure of cortical neurons to 0.5 or 2 nM CTX 3C indicated that at these concentrations the toxin did not modify the amplitude or the frequency of mEPSCs (left and right panels, respectively). Moreover, the kinetics of mEPSC were not affected by 24 hours treatment of cortical neurons with 2 nM CTX 3C (Supplementary Figure 3C).

**Chronic treatment of cortical neurons with 5 nM CTX 3C reduces the levels of AMPA and NMDA receptors through TTX-sensitive sodium channels**

The decrease in mEPSCs amplitude after a chronic neuron exposure to CTX 3C suggested changes in postsynaptic glutamate receptors. Indeed, Arc-mediated endocytosis of surface glutamate receptors is a key part of the homeostatic response to elevated activity,[46,47] therefore it seemed likely that the chronic CTX 3C treatment would have consequences in glutamate receptors expression. In order to confirm this hypothesis, the effect of a 24 h exposure of cortical neurons to 5 nM CTX 3C in the expression of AMPA and NMDA receptors was evaluated. Thus, cortical neurons were exposed for 24 hours to three different conditions: 5 nM CTX 3C alone, 5 nM CTX 3C + 0.5 µM TTX and 0.5 µM TTX alone was used as a positive control. When combined with CTX 3C, TTX was added 10 minutes prior to the addition of CTX 3C in order to ensure that $Na_v$ channels were blocked. TTX alone was used as a positive control since a chronic treatment with this $Na_v$ channels blocker has been shown to enhance the expression of AMPA and NMDA receptors.[29] As illustrated in Figure 6, pretreatment of cortical neurons with CTX 3C, induced a decrease in the NMDA receptor subunits NR2A and NR2B and in the AMPA receptor subunit GluR2/3. As shown in Figure 6A, protein levels of NMDA

receptor subunits NR2B were decreased 24 h after CTX 3C treatment by 16.9 ± 5.5% versus control cultures ($p < 0.01$). Conversely, activity blockade with TTX increased total levels of NR2B protein by 25.5 ± 11.4% ($p < 0.05$) and when CTX 3C and TTX were co-incubated, the band intensity of NR2B antibody was enhanced by 32.3 ± 11.4% ($p < 0.05$) indicating that the effect of TTX prevailed to that of CTX 3C and, therefore, the effect of CTX 3C on synaptic plasticity in cortical neurons is likely mediated by functional tetrodotoxin-sensitive sodium channels. As shown in Figure 6B, the protein levels of the NMDA receptor subunit NR2A were also decreased 24 h after CTX 3C treatment by 30.4 ± 17.0 % respect to control ($p < 0.05$). However, activity blockade with TTX or a co-incubation of TTX and CTX 3C did not affect NR2A levels compared to control conditions, probably because 24 hours treatment of cortical neurons with TTX alone did not affect NR2A levels. As shown in Figure 6C, protein levels of the AMPA receptor subunits Glur2/3 were decreased 24 h after CTX 3C treatment by 21.3 ± 8.5% ($p < 0.05$). This effect was prevented by the simultaneous treatment of cortical neurons with CTX 3C and 0.5 μM TTX. Moreover, treatment with TTX alone increased the expression of this protein by 42.9 ± 14.1% ($p < 0.05$). Altogether, these results indicate that although TTX and CTX 3C bind to different sites of sodium channels, both compounds produce opposite effects on the expression of NMDA and AMPA receptors indicating a bidirectional homeostatic compensation at the level of receptor surface expression. Furthermore, in all cases blockade of sodium channels with TTX prevented the decrease in the level of NMDA and AMPA receptor surface expression produced by CTX 3C, again supporting the idea that the chronic effects of CTX 3C on synaptic plasticity in cortical neurons are primarily mediated by its interaction with open sodium channels.

## DISCUSSION

Ciguatera is a widespread and severe form of food poisoning that causes clinically important long term neurological features that are believed to be a direct consequence of the interaction of CTX with sodium channels.[3,4,6] Although ciguatera poisoning is rarely fatal, morbidity may be high, and in some cases symptomatology, that is neurologic in more than 80% of cases, may be prolonged for several months or even years.[8] Neurological features of ciguatera

food poisoning include peripheral sensory or motor symptoms and central symptoms such as severe or prolonged distressing headache, confusion, memory disturbance, convulsions and coma.[8] Some authors argue that the unique effects of ciguatoxins on sodium channels do not fully explain the wide range of neurological symptoms elicited by this toxin,[48] however, the chronic effect of ciguatoxins on sodium channels and its involvement in other underlying mechanisms leading to chronic perturbations in activity has not been elucidated in central neurons so far. Therefore, in order to better understand the molecular basis of the neurological symptoms of ciguatera, it is important to determine the effects that a chronic CTX treatment represents to central neurons.

Immediate early genes act as messengers coupling short-term neuronal activity with changes at the level of gene transcription.[49] Our data clearly shows that CTX 3C enhanced cortical network activity in a sodium channel- dependent manner as evidenced by an increase in the mRNA level of Arc and Egr1 that was prevented by the previous blockade of sodium channels with TTX. Egr1 is a regulatory transcription factor IEG and has a strong influence on long-term cellular homeostasis by regulating the expression of other genes.[37] On the other hand, Arc is a direct effector IEG that is induced as soon as 5 min after neuronal activity stimulation and its levels remain upregulated for about 8 h.[36] Arc has a wide range of cellular functions including those related to synaptic modifications that underlie synaptic plasticity.[18,49] In this sense, Arc participates in the regulation of homeostatic synaptic scaling of glutamate receptors following chronic activity manipulations.[38,50]

We have recently described the acute effects of CTX 3C on voltage-gated sodium channels in cortical neurons.[16] Interestingly, here, the ability of CTX 3C to shift the activation curve of sodium channels to more hyperpolarizing potentials leading to membrane depolarization of cortical neurons was maintained 24 hours after CTX 3C exposure. The activation threshold of sodium currents to more negative potentials was similarly affected by an acute or by a chronic treatment of cortical neurons with 5 nM CTX 3C that shifted the activation curves of sodium channels to the left by about 26 mV after 5 min exposure and by 18 mV after 24 hours exposure. Furthermore, acute or chronic exposure of cortical neurons to 5 nM CTX 3C decreased peak $I_{Na}$ by 56 % and 63%, respectively. These results indicate that the ability of CTX 3C to activate

sodium channels is maintained after a chronic exposure of cortical neurons to the toxin. Moreover, although spiking activity in neurons treated for 24 hours with CTX 3C was significantly decreased compared to control conditions, it was not completely abolished as observed after bath application of CTX 3C to cortical neurons during 5 minutes (companion paper). The same occurred with the depolarization observed after a chronic exposure of cortical neurons to CTX 3C that accounted for about 8 mV against the stronger depolarization elicited by acute bath application of the toxin. However, after 24 hours of toxin exposure the mixed sPSCs of CTX 3C treated neurons did not differ from those of control neurons, suggesting a restoration in neuronal activity after 24 hours since an acute exposure of cortical neurons to CTX 3C increased mixed sPSCs amplitude after toxin administration (companion paper). In theory, if a compensatory mechanism occurs it would be expected that the firing activity should return to basal levels. In this direction, depolarization is an important signal to trigger homeostatic plasticity in central neurons.[17] The fact that membrane potential and electrical activity were only partially restored when the treatment was chronic indicated that neurons used compensatory mechanisms in an attempt to return neuronal activity into normal levels but probably neurons need more than 24 hours to fully restore their basal firing activity and membrane potential.

In order to avoid falling silent or runaway excitation, cortical networks use different homeostatic mechanisms that can regulate synaptic strength or neurotransmitter function in neurons. One of these mechanisms is synaptic scaling, which produces an up-regulation or down-regulation of the amplitude of all of their excitatory synapses in an activity-dependent manner.[26,27] In this sense, pharmacological manipulations of neuronal activity induce compensatory changes in the strength of glutamatergic synapses. Decreased activity (for example due to the blockade of action potential generation with TTX) enhances the strength of excitatory synapses and is reflected by an increase in the amplitude of mEPSCs.[26,27,29,50,51] On the contrary, when neuronal activity is heightened homeostatic mechanisms operate down-regulating the strength of all excitatory synapses[44] by removing glutamate receptors from synapses[27] which is reflected by a reduction in mEPSCs amplitude.[17,26] However, as far as we know, there is no evidence of whether a chronic activation of sodium

channels, which in theory means the opposite manipulation than treating cells with TTX (CTX activates sodium channels whereas TTX blocks these channels), mediates synaptic scaling in an opposite manner than TTX does. Here, the amplitudes of mixed AMPA and NMDA mEPSCs were significantly decreased in neurons treated with 5 nM CTX 3C for 24 hours when compared to control neurons, indicating that glutamatergic synaptic strength was scaled down by chronic CTX 3C treatment. Possible signals that transduce high levels of activity into a reduction in mEPSC amplitude are changes in firing rate, changes in glutamate receptor activation and changes in neuronal depolarization.[17,29,30] Taking into account that this is the first time that a downscaling of mEPSCs is achieved by a sodium channel activator, since the vast majority of pharmacological manipulations use bicuculline to downscale mEPSCs, we hypothesize that the CTX 3C induced depolarization, which is maintained after 24 hours of treatment, may have contributed to this effect. In this sense, it has been previously suggested that depolarization alone, in the absence of action-potential generation, is sufficient to down-regulate the amplitude of mEPSC[17] and, for example, a 24 h depolarizing stimulus with KCl produces a decrease of AMPA receptor levels in rat cortical neurons.[52]

The electrophysiological evidence showing that CTX 3C induced synaptic scaling in cortical neurons and suggesting down-regulation of glutamate receptors was confirmed at the biochemical level since protein levels of AMPA and NMDA receptors were decreased after CTX 3C treatment. These results are in full agreement with other studies reporting that chronically elevated network activity (by picrotoxin), decreased the levels of the NMDA receptor subunits NR2A and NR2B and the levels of the AMPA receptor subunits GluR1 and GluR2.[38] Also, in agreement with the results described here in cortical neurons, activity blockade with TTX increased the total protein levels of the NMDA receptor subunit NR2B without affecting the levels of the NR2A subunit in hippocampal neuronal cultures.[38] In that study, 48 h activity blockade with TTX did not increase the levels of AMPA receptor subunits.[38] However, in our study the levels of the AMPA receptor subunits GluR2/3 were increased in neurons treated with TTX for 24 h which is in agreement with other report indicating that a 24 h of activity blockade with TTX in cortical cultured neurons increased AMPA receptors accumulation as revealed by immunocytochemistry

experiments.[53] Overall, the effects of CTX 3C on excitatory transmission, including the reduction in the amplitude of mEPSCs that was consistent with a decrease in the levels of glutamate receptors, are supported by the enhancement of neuronal activity produced by CTX 3C evidenced by an induction of Arc mRNA. In fact, it is known that, in order to normalize excitatory drive under heightened levels of network activity, Arc mRNA levels increase and Arc mRNA is made into the protein Arc, which interacts with endophilins of the postsynaptic endocytic machinery reducing cell surface levels of glutamate receptors,[38,46,50,54,55] a mechanism that can explain the chronic effects of CTX 3C in cortical neurons.

Although most of the effects of ciguatoxins in humans can be explained by peripheral mechanims, ciguatera patients also suffer from a wide range of symptoms with central origin such as headache, fatigue, weakness, difficulty sleeping, dizziness, lack of concentration, word-finding difficulty, reduced mental capacity and unsteadiness, memory disturbance and convulsions.[2,8] Interestingly, the physiopathology of some of these symptoms can be associated to the chronic effects of CTX 3C in cortical neurons. In this sense, Arc and Egr1 play important roles in synaptic plasticity and memory functions,[37,56] therefore the finding that CTX 3C enhances the mRNA expression of these IEGs could be associated with the memory dysfunction that can appear in some cases of ciguatera.[8] On the other hand, chronic pain is a common symptom of ciguatera.[8] While peripheral and spinal sensitization contribute to early phases of chronic pain, late and persistent changes in cortical regions are necessary to maintain chronic pain.[57,58] Here, the increase in the frequency of mEPSCs in CTX 3C treated neurons indicates an increase in the release of glutamate vesicles and this presynaptic alteration is known to be associated with chronic pain.[59,60] Furthermore, other neurological alterations that are common in ciguatera food poisoning such as fatigue, weakness, depression and memory loss, are related to alterations in synaptic transmission resulting from decreased excitatory activity[62,63] that, here, was reflected by a decrease in mEPSCs amplitude and a decrease in the protein levels of glutamate receptors.

Understanding the neuronal behavior after long term exposure to ciguatoxins may help to better understand the neurological symptoms of the threatening

food poisoning ciguatera. In this direction, the results presented in this work indicate that ciguatoxin enhances neuronal activity through voltage-gated sodium channels and in order to prevent over-excitation neurons use long term homeostatic mechanisms that tend to restore neuronal activity to basal levels.

**Funding Sources**

The research leading to these results has received funding from the following FEDER cofunded-grants. From CDTI and Technological Funds, supported by Ministerio de Economía y Competitividad, AGL2012-40185-CO2-01 and Consellería de Cultura, Educación e Ordenación Universitaria,GRC2013-016, and through Axencia Galega de Innovación, Spain, ITC-20133020 SINTOX. From CDTI under ISIP Programme, Spain, IDI-20130304 APTAFOOD.From the European Union's Seventh Framework Programme managed by REA–Research Executive Agency (FP7/2007-2013) under grant agreement 312184 PHARMASEA.

**Supporting Information**

This information is available free of charge via the Internet at http://pubs.acs.org/.

**Figure S1. Effect of 24 hours exposure of cortical neurons to 0.5 nM or 2 nM CTX 3C on Arc and Egr1 mRNA levels.** Relative quantification of the effect of 0.5 and 2 nM CTX 3C on Arc, and Egr1 mRNAs by real time PCR. RQ: relative quantification using the ribosomal protein coding mRNA RPL13A as housekeeper. Relative fold expression (RQ) was determined by $2^{-\Delta\Delta CT}$. The error bars display the calculated maximum (RQ Max) and minimum (RQ Min) expression levels that represent standard deviation of the mean expression level (RQ value) with a confidence < 0.05. Each treatment was analysed in triplicate and three experiments were done.

**Figure S2. Effect of 24 h exposure of cortical neurons to 0.5 nM or 2 nM CTX 3C on neuronal spiking and resting membrane potential. A,** Average results for the effect of a chronic exposure of cortical neurons to 0.5 nM and 2 nM CTX 3C in the frequency (left panel) and amplitude (right panel) of spontaneous neuronal spikes when $V_m$ was permitted to fluctuate freely. **B,**

Average results showing the effect of a 24 hours exposure of cortical neurons to 0.5 nM or 2 nM CTX 3C on membrane potential (left panels). The depolarizing effect of 2 nM CTX 3C on membrane potential was reversed by bath application of 0.5 µM TTX during the recording (right panel). The number of cells tested is indicated in parentheses.* $p < 0.05$.

**Figure S3. A 24 hours exposure of cortical neurons to 0.5 nM or 2 nM CTX 3C did not affect the frequency nor the amplitude of mEPSCs. A,** representative traces of mEPSCs recorded in control neurons (upper panel) and in neurons treated for 24 h with 0.5 nM CTX 3C (middle panel) or 2 nM CTX 3C (lower panel). **B,** Average results for the effect of a 24 hours exposure of cortical neurons to 0.5 nM or 2 nM CTX 3C on the frequency (left panel) and amplitude (right panel) of mEPSCs. The number of cells tested is indicated in parentheses. **C,** averaged traces of mEPSCs in control neurons and in neurons treated for 24 h with 2 nM CTX 3C obtained from 5 different cells in each condition. * $p < 0.05$.

## ABBREVIATIONS

CTX, ciguatoxin; mEPSCs, miniature excitatory postsynaptic currents; Nav channels, voltage-dependent sodium channels; TTX, tetrodotoxin; GluRs, postsynaptic glutamate receptors; BIC, bicuculline; APV, D(-)-2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; $K_V$, voltage gated potassium channels; sPSC, spontaneous postsynaptic current, $V_m$, membrane potential; NR2A, NMDA receptor subunit 2A; NR2B, NMDA receptor subunit 2B.

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**Table 1. List of antibodies and primer pairs used in western blot and qPCR experiments, respectively.**

| Antibody | Immunogen | Host | Dilution | Source |
|---|---|---|---|---|
| **NMDA 2A** | C-terminal fusion protein rat NMDAR2A aa.1253−1391 | rabbit | 1:1000 | Millipore |
| **NMDA 2B** | NMDAR2B aa. 892−1051 | mouse | 1:500 | BD Bioscience |
| **GluR2/3** | GluR2/3 aa 864-883 | rabbit | 1:500 | Millipore |
| **Actin** | C-terminal actin fragment, clone C4 | mouse | 1:3000 | Millipore |

| Target[1] | Gene Bank Accession[2] | Sequence[3] | Product length | Melting temperature[4] |
|---|---|---|---|---|
| **ARC** | NM_018790.3 | GGTGAGCTGAAGCCACAAATG<br>GTATGAATCACTGCTGGGGGC | 80 | 60.07<br>61.09 |
| **EGR1** | NM_007913.5 | AGCAGCGCCTTCAATCCTCA<br>TCTCCACCATCGCCTTCTCA | 109 | 62.19<br>60.61 |
| **RPL13A[5]** | NM_009438.5 | GCAAGTTCACAGAGGTCCTCA<br>AGGCATGAGGCAAACAGTCTTTA | 72 | 60.49<br>59.18 |

1 Official gene symbol.

2 Sequences used as templates for primer design.

3 Sequences are 5'->3'. For each target, the first sequence correspond to the forward primer and the second to the reverse primer.

4 According to NCBI-primer blast.

5 Housekeeping gene

## FIGURE LEGENDS

**Figure 1. CTX 3C effects on Arc and Egr1 mRNA levels.** Relative quantification of the effect of 5 nM CTX 3C on Arc, and Egr1 mRNAs by real time PCR. **A,** RQ in control neurons, in neurons treated during 30 minutes with 5 nM CTX 3C, and in neurons simultaneously treated with 5 nM CTX (30 min) in the presence of 0.5 μM TTX, added to the culture medium 10 minutes before CTX 3C. Note that preincubation of cortical neurons with TTX reversed the mRNA increase produced by CTX 3C. **B**, The same experiment was performed and the increase in Arc and Egr1 levels induced by 30 minutes exposure of cortical neurons to 5 nM CTX 3C was not blocked when TTX was added after CTX 3C. RQ: relative quantification using the ribosomal protein coding mRNA RPL13A as housekeeper. Relative fold expression (RQ) was determined by $2^{-\Delta\Delta CT}$. Error bars display the calculated maximum (RQ Max) and minimum (RQ Min) expression levels that represent standard deviation of the mean expression level (RQ value) with a confidence < 0.05. Each treatment was performed in triplicate.

**Figure 2. Effect of 24 h CTX 3C on voltage-gated potassium and sodium currents in cortical neurons.** Neurons were voltage clamped at a membrane holding potential ($V_m$) of -60 mV and $I_K$ was evoked by a 200 ms depolarizing pulse from $V_m$ to + 75 mV in 15 mV steps (inset). **A and B,** representative recording from a single cortical neuron in control conditions or after 24 h exposure to 5 nM CTX 3C, respectively. Whole-cell currents consisted of voltage-gated sodium currents (downward deflection in the current recording) and voltage-gated potassium currents. Note that 24 h exposure of cortical neurons to 5 nM CTX 3C did not affect $I_K$, however it decreased sodium-current amplitude $I_{Na}$. **C,** current-voltage relationship for $I_K$ recorded in control conditions and after 24 h exposure of cortical neurons to 5 nM CTX 3C. **D,** pooled results of the effect of 5 nM CTX 3C on the peak amplitude of sodium currents (left panel), measured at -15 mV, and on sodium current activation (left panel). The effect of 24 h treatment of cortical neurons with 5 nM CTX 3C was measured by plotting the percent inhibition of the peak $I_{Na}$ and the voltage activation threshold of $I_{Na}$. In CTX 3C-treated cultures peak $I_{Na}$ amplitude was significantly reduced

and the activation voltage of $I_{Na}$ was shifted towards more negative values. The number of cells tested is indicated in parentheses. **p < 0.01, ***p < 0.001.

**Figure 3. Effect of 24 h exposure of cortical neurons to 5 nM CTX 3C on neuronal spiking and resting membrane potential. A,** representative voltage traces of a neuron showing spontaneous firing activity when $V_m$ was permitted to fluctuate freely in control neurons (upper panel) and in neurons treated for 24 h with 5 nM CTX 3C (lower panel). The presence of 5 nM CTX 3C for 24 h depolarizes the membrane potential (indicated by arrows) and decreases the frequency and amplitude of spontaneous spikes. **B,** pooled results for the effect of a 24 hours exposure of cortical neurons to 5 nM CTX 3C on the frequency (left panel) and amplitude of spontaneous neuronal spikes (middle panel) and on resting membrane potential (right panel). The number of cells tested is indicated in parentheses. * p < 0.05, **p < 0.01.

**Figure 4. Effect of 24 h exposure of cortical neurons to 5 nM CTX 3C on mixed spontaneous postsynaptic currents. A,** detailed traces of mixed sPSCs recorded in control neurons and in neurons treated with 5 nM CTX 3C during 24 h at a holding potential of -80 mV. **B,** pooled results for the effect of a 24 hours exposure of cortical neurons to 5 nM CTX 3C on the frequency, amplitude and area of mixed spontaneous postsynaptic currents. The number of cells tested is indicated in parentheses.

**Figure 5. Twenty four hours exposure of cortical neurons to 5 nM CTX 3C increased the frequency but decreased the amplitude of miniature excitatory postsynaptic currents. A,** representative traces of mEPSCs recorded in control neurons (upper panel) and in neurons treated for 24 h with 5 nM CTX 3C (lower panel). **B,** average histograms showing the effect of a 24 hours exposure of cortical neurons to 5 nM CTX 3C on the frequency and amplitude of mEPSCs (left and right panels, respectively). The number of cells tested is indicated in parentheses. **C,** averaged traces (left panel) and scaled average traces (right panel) of mEPSCs in control neurons and in neurons treated for 24 h with CTX 3C obtained from 7 different cells in each condition. * p < 0.05.

**Figure 6. Twenty four hours treatment of cortical neurons with 5 nM CTX 3C induced changes in glutamate receptors expression. A,** representative

experiment showing western blot bands for the NMDA receptor NR2B subunit levels in control and treated neurons (left panel) and the corresponding quantitative histograms showing NR2B expression in control neurons and in neurons treated for 24 hours with 5 nM CTX 3C alone, in neurons treated with 5 nM CTX 3C in the simultaneous presence of 0.5 μM TTX and in neurons treated with 0.5 μM TTX alone (right panel). **B**, The same treatments were performed to evaluate their effect on the expression of the NMDA receptor subunit NR2A. Representative western blot bands showing NR2A levels are shown in the left panel. The corresponding quantification of the western blot bands intensities are shown in the right. 24 hours exposure of cortical neurons to 5 nM CTX 3C decreased NR2A levels but TTX alone or the simultaneous treatment of cortical neurons with CTX and TTX did not modify NR2A levels in cortical neurons (right panel). **C**. Representative western blot bands showing the effect of the same treatments on the levels of the AMPA receptor subunits GluR2/3 (left panel). Quantification of western band intensities (right panel) indicates that 24 hours treatment of cortical neurons with 5 nM CTX 3C decreased GluR2/3 levels in cortical neurons, an effect opposite to that of TTX and which was prevented when the CTX treatment was performed in the presence of TTX. Data were obtained from at least 3 independent experiments from 3 different neuronal cultures. * $p < 0.05$, **$p < 0.01$ (versus control); # $< 0.05$, ## $p < 0.01$ (versus CTX 3C).

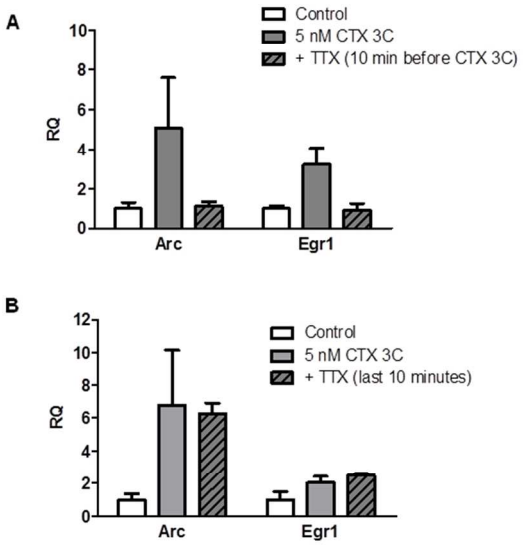


Figure 1

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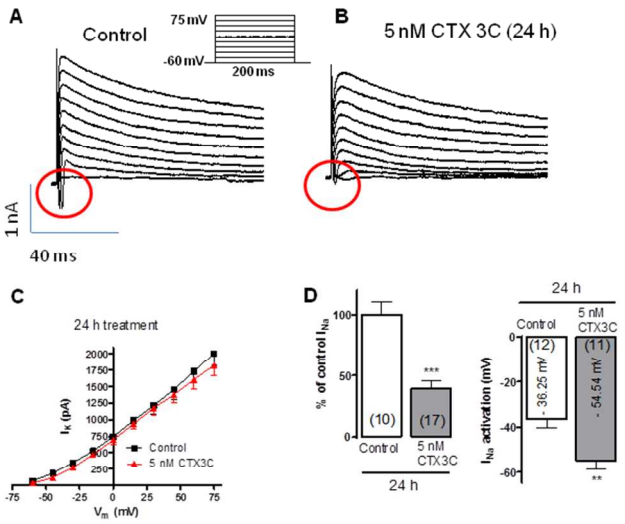


Figure 2

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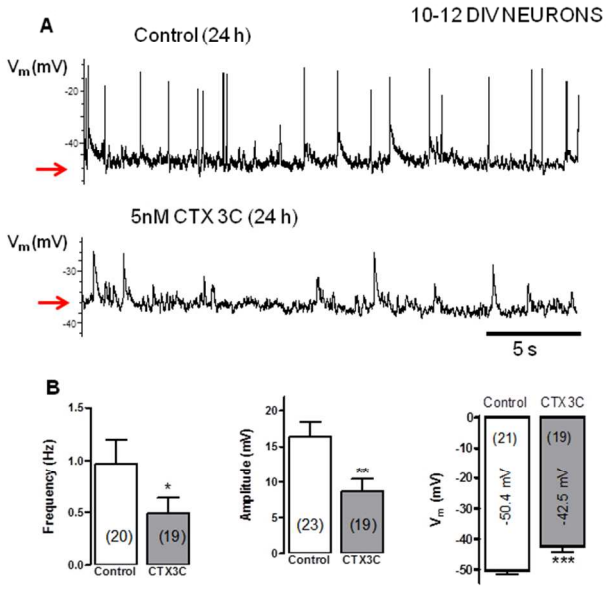


Figure 3

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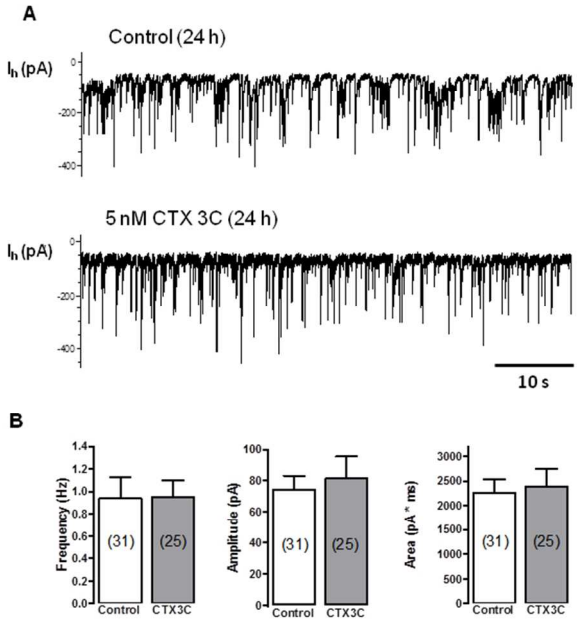


Figure 4

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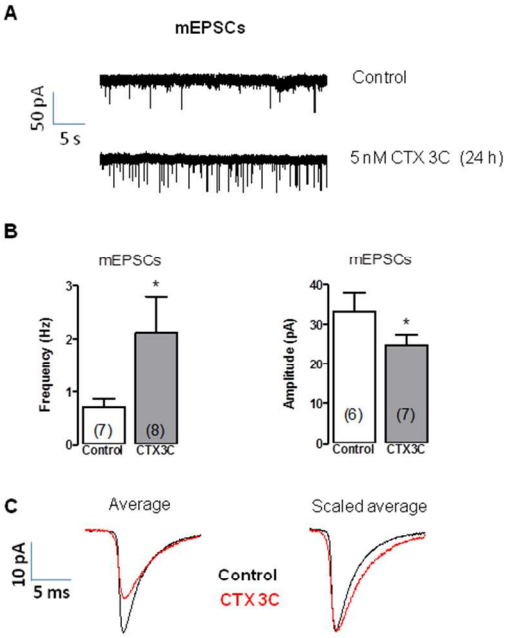


Figure 5

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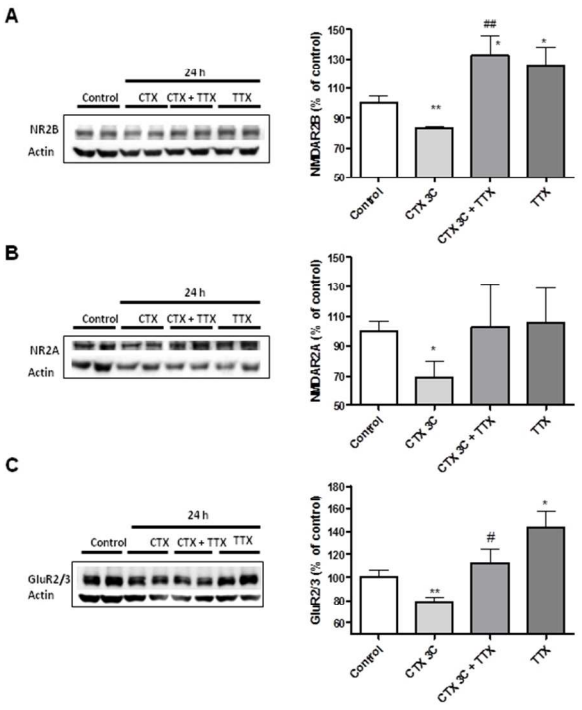


Figure 6

254x190mm (96 x 96 DPI)