Synthetic ciguatoxin CTX 3C induces a rapid imbalance of neuronal excitability

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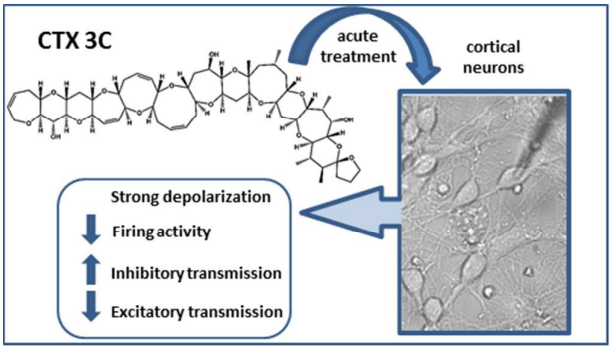

Abstract

Ciguatera is a human global disease caused by the consumption of contaminated fish that have accumulated the sodium channel activator toxins ciguatoxins (CTX). Symptoms of ciguatera include neurological alterations such as paraesthesiae, dysaesthesiae, depression and heightened nociperception, among others. An important issue to understand these long term neurological alterations is to establish the role that changes in activity produced by CTX 3C represent to neurons. Here, the effects of synthetic ciguatoxin CTX 3C on membrane potential, spontaneous spiking and properties of synaptic transmission in cultured cortical neurons of 11-18 DIV were evaluated by using electrophysiological approaches. CTX 3C induced a large depolarization that decreased neuronal firing and caused a rapid inward tonic current that was primarily GABAergic. Moreover, the toxin enhanced the amplitude of miniature postsynaptic inhibitory currents (mIPSCs) while it decreased the amplitude of miniature postsynaptic excitatory currents (mEPSCs). The frequency of mIPSCs increased while the frequency of mEPSCs remained unaltered. We describe for the first time that a rapid membrane depolarization caused by CTX 3C in cortical neurons activates mechanisms that tend to suppress electrical activity by shifting the balance between excitatory and inhibitory synaptic transmission towards inhibition. Indeed, these results suggest that the acute effects of CTX on synaptic transmission could underlie some of the neurological symptoms caused by ciguatera in humans.

Introduction

Neuronal homeostasis plays an essential role in the formation, maintenance and modification of neuronal circuits and provides neurons a reliable way to adapt to changes in the levels of activity.[1,2] Neuronal changes in intracellular sodium concentration control action potential generation and mediate forms of synaptic plasticity that depend on neuronal firing.[3,4] In this sense, marine neurotoxins acting on voltage gated sodium channels (VGSC) have been very useful in the study of the role of excitability in various synaptic preparations.[5] The VGSC activator brevetoxin (PbTx), has been shown to increase N-methyl-D-aspartate receptor (NMDA) function and promote neurite growth in immature cortical neurons at concentrations that do not modify the intracellular calcium concentration.[4] Among the marine toxins that activate sodium channels, CTXs are the most potent activators of VGSC, causing cell membrane depolarization at rest, by increasing sodium influx, leading to persistent neurological changes in humans intoxicated by contaminated fish containing CTXs.[6-12] The physiological consequence of the binding of ciguatoxins to sodium channels is an initial increase in cellular excitability, which results in spontaneous and repetitive firing of action potentials, followed by a decrease in excitability as the membrane further depolarizes.[13] However, most of the studies to date have focused on the effects of ciguatoxin in the excitability of peripheral nervous system and not in central neurons.

An important issue to understand the neurological consequences of ciguatera food poisoning in humans is to establish the role that changes in activity elicited by CTX could represent to central neurons. The neurological sequel of ciguatera fish poisoning in humans usually resolves within weeks of onset, however, some nervous symptoms may persist for months or even years.[14] In humans, symptoms of ciguatera fish poisoning manifest with paraesthesiae, dysaesthesiae, and heightened nociception, as well as sensory abnormalities which include subjective features of metallic taste, pruritus, arthralgia, myalgia and dental pain.[14] Interestingly, alterations in inhibitory transmission are involved in altered body temperature and ataxia,[15] both observed in ciguatera.[11,16] Moreover, fatigue, weakness and depression which result from decreased excitatory activity or increased inhibitory activity or both[17] are common in ciguatera food poisoning.[14] Therefore we hypothesized that some of those neurological effects may be related with alterations in synaptic homeostasis or perturbations in the global network transmission.

Although the clinical consequences of ciguatera are long lasting, firstly it is necessary to establish the effects of an acute exposure of central neurons to CTX in order to set the basis to future works. In this direction, glutamate receptors can be regulated by rapid (5 min) membrane trafficking, modulating synaptic transmission in response to rapid activation.[18] Indeed, $GABA_A$ receptors can be also modulated in a short time scale after neuronal depolarization.[19] and ciguatoxin has been previously shown to decrease GABA uptake and increse GABA release in rat brain synaptosomes.[20] Therefore, the main objective of this work was to analyze the acute effect of CTX 3C on spontaneous electrical activity and synaptic transmission in cortical neurons.

Altogether the results presented here indicate that the depolarization caused by the sodium channel activator CTX 3C reduces neuronal firing rate and shifts the balance between inhibitory and excitatory neurotransmission towards inhibition. These results constitute a first approach to better understand the cellular mechanisms that might lead to the neurological symptomatology of human ciguatera food poisoning and thus provide a basis for future studies.

Materials 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 (authorization code: AE-LU-002/07/01/03/LBL4) and comply with European legislation on the use and management of experimental animals. Primary cortical neurons were obtained from embryonic day 16–18 Swiss mice as previously described.[21] Briefly, cerebral cortices were removed and dissociated by mild trypsinisation, followed by mechanical trituration in a DNAse solution (0.004% w/v) containing a soybean trypsin inhibitor (0.05% w/v). The cells were suspended in Neurobasal Medium supplemented with 1% B-27 supplement (Invitrogen), 5 mM L-glutamine and 1% penicillin/streptomycin. Cell suspension was seeded in 12 well plates precoated with poly-D-lysine and the cell culture was kept in a 95% air, 5% CO_2 atmosphere at 37 ºC. Culture medium was replaced every 3-4 days. All data were obtained in parallel from drug-treated and age-matched sister control cultures.

Electrophysiology

Whole cell patch-clamp recordings, achieved by gentle mechanical suction of the membrane patch, were performed on cortical neurons, between 11-18 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, 1.5mm) and the tip resistance was 5-10MΩ. Only recordings with stable access resistance and 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) while intracellular pipette solutions contained (in mM): 150 KCl, 2 $MgCl_2$, 5 HEPES, 1.1 EGTA and 2 Na_2ATP (pH 7.2). Data were rejected if the initial resting potential was more depolarized than -50 mV.

In order to evaluate the effect of CTX 3C in neuronal spiking and resting membrane potential miniature synaptic events were recorded in voltage-clamp mode at a holding potential of -80 mV. Events were counted and analyzed over a 1 minute period immediately before (control) and over a 1 minute period after 4 minutes of compound exposure. Miniature excitatory postsynaptic currents were recorded in the presence of the $GABA_A$ receptor antagonist, bicuculline (BIC) 20 μM, and the VGSC blocker, tetrodotoxin (TTX) at 0.5 μM to block action potentials. Miniature inhibitory postsynaptic currents were recorded in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM), a NMDA receptor antagonist, D(-)-2-amino-5-phosphonopentanoic acid (APV, 100 μM) and 0.5 μM TTX.[22] 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 or mIPSC for a given experimental condition, all the events from every neuron recorded in one minute and in each condition were averaged.[23] Tonic current (I_{hold}) was measured respect to the average baseline level of all the events detected in each experimental condition.

Toxins and drugs used

The standard of CTX 3C was synthesized by Dr. Masahiro Hirama following previously described procedures[24-26] and dissolved at a concentration of 10 μM in DMSO. Following dilutions were performed in deionized 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. Statistical comparisons were made by paired Student's t test. p values < 0.05 were considered statistically significant.

Results

The main mechanism of action of CTXs is the activation of VGSCs at hyperpolarizing membrane potentials causing cell membrane depolarization at rest and eventually irreversible blockade of neurotransmitter release.[5 27-30] The effect of synthetic CTX 3C on VGSCs has been described in several cellular models including mouse taste cells, cerebellar granule neurons, cortical neurons and sodium channel cell lines.[27, 28, 31, 32] In addition, CTX 3C can activate TTX resistant sodium channels and this effect has been suggested to explain the neurological symptoms induced by CTXs in humans such as hyperalgesia and allodynia among others.[29] Moreover, in immature cortical neurons, 48 hours exposure to neurotoxins belonging to the group of sodium channel activators modified activity-dependent synaptic plasticity by increasing NMDA receptor function .[4]

In order to determine the toxin concentration that exerts an effect in cortical neurons and to provide a basis for further experiments, the effect of several concentrations of CTX 3C on VGSC was first evaluated. Voltage-dependent sodium currents were elicited in cortical neurons by applying a series of 25 ms depolarizing pulses (voltage steps), in 5 mV increments, from a holding potential of -100 mV.[21] As shown in Figure 1A, the activation of sodium channels was shifted in the hyperpolarizing direction after bath application of CTC 3C. Despite 0.1 nM CTX 3C did not affect the activation of voltage gated sodium channels (data not shown), bath application of 1 and 5 nM CTX 3C,

shifted the activation threshold of sodium currents to more negative potentials, from -50.0 ± 4.7 mV (n = 5) in control conditions to -64.0 ± 3.6 mV (n = 5; p = 0.02) at 1 nM CTX 3C and -76.0 ± 2.9 mV (n = 5; p = 0.0008) at 5 nM CTX 3C (Figure 1B). Furthermore, sodium current amplitude was decreased by ciguatoxin as previously described.[29, 32-34] Representative voltage-dependent sodium currents at a test potential of -10 mV in the absence and presence of 5 nM CTX 3C are shown in Figure 1C. A concentration of 5 nM CTX 3C decreased peak I_{Na} by 55.8 ± 8.4% ($p < 0.05$; n = 5) as shown in Figure 1D.

CTX 3C depolarizes and silences cortical neurons

An increase in neuronal excitability resulting in membrane depolarization and repetitive action potential discharges elicited by CTXs has been previously described using several experimental preparations such as frog motor nerve terminals,[9] neuroblastoma cells[20] and mammalian dorsal root ganglion neurons.[35] However, the effects of these CTXs have been described on evoked action potentials and there is only one report describing the effect of the Pacific CTX-1 (P-CTX1) on spontaneous activity in rat parasympathetic neurons.[7]

In order to elucidate the effects of CTX 3C in membrane excitability and membrane potential (V_m), whole-cell current clamp experiments were performed in 11-18 div cortical neurons, which exhibited spontaneous firing activity at rest. A threshold of 8 mV was established for considering neuronal spikes. Bath application of 5 nM CTX 3C, but not 1 nM CTX 3C, to isolated cortical neurons caused a rapid membrane depolarization maintained over several minutes resulting in a decrease in spontaneous firing which finally ceased in all the fourteen cells tested. Typical effects of 1 nM CTX 3C or 5 nM CTX 3C on V_m and excitability in cortical neurons are shown in Figure 2A and 2B, respectively. As shown in the left panel of Figure 2C, the spontaneous firing rate of cortical neurons was 1.6 ± 0.6 Hz in control conditions and 1.5 ± 0.5 Hz (n = 3; p = 0.5) in the presence of 1 nM CTX 3C and the amplitude of spontaneous spikes was 16.5 ± 3.1 mV in the absence of toxin and 13.8 ± 1.5 mV in the presence of 1 nM CTX 3C (n = 3; p = 0.2). However, as shown in the left panel of Figure 2D, the spontaneous firing rate was significantly decreased from 2.3 ± 1.8 Hz in control conditions to 0.17 ± 0.1 Hz (n = 10; p = 0.0006) in the presence of 5 nM CTX 3C. This observation is in contrast with other reports where CTXs increased repetitive firing in other cellular preparations,[7,35,36] a fact that could be explained by the high prevalence of the inhibitory

neurotransmitter GABA in cortical neurons[37] or by the large depolarization caused by the toxin in the present study. Moreover, the amplitude of spontaneous spikes (Figure 2D, right panel) was decreased from 21.3 ± 3.8 mV in the absence of toxin to 2.6 ± 1.3 mV in the presence of 5 nM CTX 3C (n = 10; p = 0.00008).

In order to know whether the decrease in neuronal firing caused by CTX 3C was a direct consequence of the cellular depolarization elicited by the toxin, the membrane potential of cortical neurons was held at -55 mV through current injection. Under these conditions, exposure of cortical neurons to 1 nM CTX 3C did not alter the firing rate of cortical neurons (Figure 3A), however, as shown in Figure 3B, 5 nM CTX 3C decreased spontaneous firing activity and, in parallel, the current necessary to maintain the membrane potential at -55 mV was enhanced after bath application of 5 nM CTX 3C. As shown in Figure 3C, mean values for spiking frequency (left panel) were 1.8 ± 0.6 Hz in control conditions and 1.9 ± 0.5 Hz in the presence of 1 nM CTX 3C (n = 4; p = 0.47) and the mean values for the amplitude of neuronal spikes (right panel) were 18.8 ± 3.3 mV in control conditions and 17.4 ± 7.7 mV in cells treated with 1 nM CTX 3C (n=4; p = 0.43). As shown in the left panel of Figure 3D, at -55 mV spontaneous firing rate was 2.1 ± 1.2 Hz in control conditions, whereas 5 nM CTX 3C reduced the firing rate to 0.3 ± 0.2 Hz (n = 5; p = 0.08), although this result did not reach statistical significance probably due to the high variability in the spiking pattern of these neurons. However, as indicated in Figure 3D (right panel), the spike amplitude was reduced from 20.4 ± 3.0 mV in control conditions to 6.2 ± 2.5 mV after bath application of 5 nM CTX 3C (n = 5; p = 0.003) indicating that the silencing effect of 5 nM CTX 3C on neuronal spiking was not only due to the depolarization.

Additionally, the effect of CTX 3C on membrane potential was studied. CTX 3C at 1 nM did not affect average resting membrane potential that was -51.3 ± 2.2 mV in control conditions and -48.2 ± 4.1 mV (n = 4; p = 0.26) in the presence of 1 nM CTX 3C (Figure 3E left panel). However, resting membrane potential was significantly depolarized from -53.7 ± 2.9 mV in control conditions to -31.7 ± 4.9 mV (n = 14; p < 0.001) in the presence of 5 nM CTX 3C (Figure 3E right panel). The depolarizing effect of 5 nM CTX 3C in cortical neurons was suppressed when 0.5 μM TTX was added to the bath before CTX 3C at 5 nM (Figure 3F). In this condition resting membrane potential was -51.6 ± 2.1 mV in the presence of TTX and -48.4 ± 2.5 mV in the simultaneous presence of TTX and 5 nM CTX 3C (n = 5), thus indicating that the

depolarizing effect of CTX 3C in cortical neurons was mainly mediated by TTX-sensitive VGSCs.

CTX 3C induces a shift in I_{hold} and increases the amplitude and area of mixed spontaneous postsynaptic currents

To investigate the mechanisms underlying the increase in the current necessary to maintain V_m at -55 mV after bath application of CTX 3C (described in Figure 3C), the effect of CTX 3C under voltage clamp conditions (holding potential, -80 mV) was analyzed. A typical effect of 5 nM CTX 3C in the holding current (I_{hold}) of a cortical neuron is shown in Figure 4A. CTX 3C elicited an increase in I_{hold} from -45.8 ± 4.7 pA in control conditions (n = 7) to -81.6 ± 17.2 pA (n = 3; $p < 0.05$) and -236.6 ± 48.8 pA (n = 7, $p < 0.001$) after bath addition of 1 nM and 5 nM CTX 3C, respectively (Figure 4B). During the course of these experiments it was clear that spontaneous synaptic currents were affected by bath application of CTX 3C and we further analyzed this effect in the following experiments.

In neurons, spontaneous synaptic events may be divided into those resulting from a spontaneous action potential in a presynaptic neuron (spontaneous postsynaptic current, sPSC) which are involved in the regulation of postsynaptic firing and synapse homeostasis and those resulting from the release of a single transmitter vesicle in the absence of presynaptic activity (miniature postsynaptic current, mPSC).[38, 39] The effect of CTX 3C on sPSCs was analyzed by quantifying the effect of the toxin in the amplitude and distribution of sPSCs.

The amplitude and area of mixed inward sPSCs were increased after bath application of CTX 3C. A detailed image of sPSCs recorded before (control) and after administration of 1 nM and 5 nM CTX 3C is shown in Figure 4C, upper and lower panel, respectively. At 1 nM, CTX 3C did not alter the frequency nor the distribution of sPSCs amplitudes (Figure 4D upper panel) while at 5 nM CTX 3C shifted the frequency distribution of sPSCs amplitudes to the right (Figure 4D lower panel), indicating an increase in the number of sPSCs with higher amplitudes. As shown in Figure 4E, at 5 nM CTX 3C increased the normalized amplitude of sPSCs (199.3 ± 34.2% of control; n = 8; p = 0.01), while the toxin at 1 nM lacked an effect. Since amplitude shifts do not necessarily mean a change in charge transfer, the area of sPSCs was also measured and it was increased by 212.1 ± 38.9% of the control area by 5 nM CTX 3C (n = 7; p = 0.008),

while at the concentration of 1 nM CTX 3C did not significantly affected the area of sPSCs as shown in Figure 4F.

CTX 3C induces a tonic current in cortical neurons that is mainly GABAergic

CTX 3C, at higher concentrations than those employed in this work, induced a large leak current (more than 400 pA) in HEK cells expressing TTX-resistant sodium channels but not in HEK cells expressing TTX-sensitive sodium channels.[29] In contrast, other authors reported that P-CTX 1 induced a large leak current only in DRG cells expressing predominantly TTX-sensitive sodium channels.[33] However, none of these reports investigated the underlying mechanism for the leak current induced by CTX, thus the implication of TTX sensitive or resistant channels in this effect is controversial. However, it is known that GABA activates a persistent tonic current in several brain regions[40] and that purified ciguatoxin increases ambient GABA levels in rat brain synaptosomes.[20]

The standard method to reveal the presence of a GABAergic tonic currents in a whole-cell recording is to apply a saturating concentration of a specific $GABA_A$ receptor antagonist like BIC.[41] Therefore, we analyzed whether the enhancement of I_{hold} by CTX 3C could be reversed by blocking TTX sensitive channels or $GABA_A$ receptors. For this purpose, a sequence of CTX 3C, TTX and the $GABA_A$ antagonist BIC were added to the bath (Figure 5A). In these conditions control I_{hold}, -56.9 ± 3.8 pA, was increased by 5 nM CTX 3C to -214.0 ± 28.5 pA (n = 4, p = 0.0007), similarly to the results obtained in the set of experiments described in the previous section. Further addition of 0.5 μM TTX caused only a small decrease in I_{hold} to -187.4 ± 31.0 pA (p = 0.27) while addition of 20 μM BIC significantly reversed I_{hold} to near control values (-77.9 ± 11.6 pA; p = 0.008 respect to TTX) indicating that the tonic current induced by CTX 3C was mainly mediated by $GABA_A$ receptors. A representative trace of this effect is shown in Figure 5B. The tonic current activated by CTX 3C was composed of three components, a fast decay followed by a fast recovery (few seconds) which ended in a stable I_{hold} level resembling the tonic current activated by GABA in cortical neurons (Supplementary Figure 1) as well as in other cellular models.[42] As shown in Figure 5C, the membrane depolarization caused by the toxin was simultaneously accompanied by a large increase in the inward tonic current, indicating that the effects of CTX 3C on membrane potential and I_{hold} were fully linked. Moreover, bath application of TTX after CTX 3C did not affect the depolarization induced by CTX 3C but bath application of BIC

returned the membrane potential and the holding current to basal levels. This is in full agreement with previous studies reporting that extrasynaptic $GABA_A$ receptors, probably activated by increased ambient GABA[20] in the presence of CTX 3C, which mediate tonic inhibition, are strongly modulated by membrane potential.[43]

It is well known that TTX binding physically blocks the flow of sodium ions through the channel, thereby preventing action potential generation and propagation,[44] however, after activation of VGSC by CTX and the consequent membrane depolarization, TTX lacked an effect either on the recovery of I_{hold} or in V_m. Therefore, we further studied whether the order of drug administration could influence the effect of CTX 3C on I_{hold}, therefore VGSC and $GABA_A$ receptors were blocked prior to the addition of CTX 3C. In these conditions, the toxin did not modify I_{hold}. As shown in Figure 5D upper panel, I_{hold} was -55.3 ± 8.5 pA, in the presence of TTX and BIC, and -61.2 ± 8.8 pA after the administration of 5 nM CTX 3C ($n = 8$; $p = 0.32$). A representative recording showing the effects of 5 nM CTX 3C on I_{hold} after blockade of $GABA_A$ receptors and VGSC is shown in Figure 5D lower panel.

CTX 3C increases the amplitude and frequency of miniature inhibitory postsynaptic currents

Since neurons maintain stable firing rates through homeostatic regulation of many aspects of neuronal excitability such as the regulation of inhibitory and/or excitatory synaptic strength,[45] the effect of the toxin on miniature inhibitory and excitatory synaptic events was analyzed. Miniature events are assumed to report synaptic function at the level of a single terminal, representing the postsynaptic response to the release of individual vesicles of neurotransmitter and can be considered a measure of the unit strength of a synapse.[46]

Firstly, mIPSCs were pharmacologically isolated as described in the material and methods section. These mIPSC in cortical neurons were GABAergic as indicated by their complete inhibition by the $GABA_A$ receptor antagonist BIC at 20 μM (Supplementary Figure 2). A representative trace showing the effect of 5 nM CTX 3C on mIPSCs is shown in Figure 6A. Bath application of CTX 3C elicited not only an inward tonic current (discussed in the previous section) but also an increase in the amplitude and frequency of mIPSCs. In these conditions, blockade of VGSC and glutamatergic transmission, CTX 3C increased the tonic current from -45.6 ± 9.0 pA in the absence of toxin to -77.9 ± 13.2 pA ($n = 5$, $p = 0.038$) in the presence of 5 nM CTX 3C (data not shown). This effect accounts for approximately 32 pA of tonic current, and

is much lower than the tonic current elicited by CTX 3C in the absence of TTX + APV + CNQX (around 190 pA). This could be explained due to the fact that the blockade of VGSC by TTX decreases the tonic GABAergic current.[47] A detailed image of mIPSCs in both conditions (control and presence of CTX 3C) as well as a histogram of the amplitudes of mIPSCs from the same cell in each condition is shown in Figure 6B, indicating an increase in the number of inhibitory events of higher amplitude in the presence of CTX 3C. Similarly, Figure 6C shows a histogram of average mIPSC amplitude from 5 neurons (the same number of events per condition were pooled), reflecting that the average population of mIPSCs with lower amplitudes decreased in the presence of CTX 3C whereas the population of mIPSCs with higher amplitudes was enhanced by the toxin. As illustrated in Figure 6D, 5 nM CTX 3C significantly increased the amplitude (148.93 ± 19.0% of control; n = 4; p = 0.04), the frequency (173.2 ± 24.1% of control; n = 4; p = 0.03) and the area (146.4 ± 17.2 of control; n =4; p = 0.04) of mIPSCs. The averaged traces for mIPSCs obtained in control conditions and after CTX 3C treatment are shown in Figure 6E.

Since both, amplitude and frequency of mIPSCs were significantly affected by CTX 3C, we conclude that the toxin potentiates GABAergic transmission through both postsynaptic and presynaptic mechanisms.

CTX 3C decreases the amplitude but not the frequency of miniature excitatory postsynaptic currents

It is known that changes in neuronal activity usually regulate inhibition and excitation in opposite directions.[45] Thus, we next examined the effect of CTX 3C on glutamatergic miniature excitatory postsynaptic currents (mEPSCs) as described in the materials and methods section. mEPSCs were confirmed by their complete inhibition in the presence of 20 μM CNQX and 100 μM APV (Supplementary Figure 3).

A representative trace showing the effect of CTX 3C on mEPSCs is shown in Figure 7A. In the presence of TTX and BIC, CTX 3C decreased the amplitude of mEPSCs. A more detailed image of mEPSCs from the same recording is shown in Figure 7B. A histogram of the amplitudes from the same cell in each condition reflected that the population of mEPSCs with lower amplitudes was enhanced in the presence of CTX 3C whereas the population of mEPSCs with higher amplitudes tended to disappear (Figure 7B, inset). As illustrated in Figure 7C, the distribution of mEPSC amplitudes recorded from 8 neurons in absence (control) and presence of CTX 3C showed a leftward shift

indicating an increase in the number of events with lower amplitude in the presence of the toxin. Plotted average mEPSCs amplitude and frequency indicated that 5 nM CTX 3C significantly decreased the amplitude and the area of mEPSC by 32.3 ± 7.8% ($p = 0.006$, $n = 8$) and 63.9 ± 12.4% ($p = 0.012$, $n = 8$), respectively, without affecting their frequency (Figure 7D). Representative traces of averaged excitatory events recorded from 8 individual neurons, as well as the CTX 3C sensitive current obtained by subtracting the remaining current in the presence of CTX 3C from the control excitatory current, are shown in Figure 7E. In this case, the normalized area decreased from 100 ± 21.8 % in control conditions to 36.1 ± 12.5 % in the presence of CTX 3C ($p = 0.012$).

Discussion and conclusions

Cortical circuits are susceptible of instability when the balance between excitation and inhibition is perturbed therefore, in order to prevent neuronal networks from runaway excitation, a homeostatic negative feedback regulation is necessary.[46] Compensatory mechanisms are generally considered to operate in a slow time course,[46] however, synaptic transmission can be regulated in a short time scale after an stimulus.[18, 19, 48] To our knowledge, this is the first study that provides direct evidence for a rapid modulation of excitatory and inhibitory transmission by the VGSC activator CTX 3C. Here, we show that the effects of CTX 3C in cortical neurons were reflected by rapid changes in membrane potential, electrical activity, GABAergic tonic current, the amplitude of mEPSCs and the frequency and amplitude of mIPSCs.

It is broadly known that ciguatoxins induce membrane depolarization in excitable cells, due to their ability to activate VGSC. As expected, in cortical neurons CTX 3C elicited a hyperpolarizing shift in the activation potential of VGSC which is a typical effect of ciguatoxins[33] also described for the synthetic toxin CTX 3C.[27, 28] Moreover, the toxin caused a decrease in the peak amplitude of sodium currents similar to the effects previously described for CTX 3C[27,28,32] and for other ciguatoxins.[49] However, the consequences of a massive depolarization by CTX 3C on neuronal transmission had never been investigated so far.

It is normally assumed that depolarization, for example that induced by an increase in intracellular sodium, is related to an increase in neuronal firing, however, in cortical neurons CTX 3C caused a large depolarization (about 20 mV) that was accompanied by a complete silencing of neuronal firing. It has been described that certain VGSC

modifiers, such as veratridine, generate depolarization block which abolishes firing by inducing persistent sodium currents.[50] However, in our study, when membrane potential was held at -55 mV, the firing properties of cortical neurons were still abolished by CTX 3C indicating that the decrease in the neuronal firing properties produced by the toxin was not only a consequence of membrane depolarization and suggested that additional mechanisms activated by the toxin were inhibiting neuronal activity. In this direction, an increase in inhibitory neurotransmission is known to decrease neuronal activity.[45]

The main inhibitory neurotransmitter receptors in the central nervous system, $GABA_A$ receptors, mediate two distinct forms of inhibition depending on their location. On the one hand, synaptic $GABA_A$ receptors mediate phasic inhibition, which is reflected as rapid inward currents that are generated by postsynaptic $GABA_A$ receptors in response to vesicular GABA release. On the other hand, extrasynaptic $GABA_A$ receptors are activated by ambient GABA and mediate tonic inhibition.[51-53] The level of tonic inhibition can rapidly change due to factors associated with intense neural activity, including increased ambient GABA, extracellular K^+ accumulation, and neuronal depolarization[19,43,47] and GABAergic tonic currents regulate neuronal excitability by setting the threshold for action potential generation.[53]

Here, CTX 3C induced a GABAergic vesicular release as evidenced by the increase in mIPSCs frequency (increase in phasic inhibition). We suggest that this effect caused an increase in ambient GABA, which in turn activated extrasynaptic $GABA_A$ receptors as indicated by the GABAergic tonic current induced by CTX 3C. In fact, ciguatoxin at 10 ng/ml has been previously shown to increase ambient GABA levels in a concentration-dependent manner in rat brain synaptosomes and this effect was mediated through an increase in GABA release and a decrease in GABA uptake.[20] Therefore, the results presented here suggest that the activation of GABAergic tonic currents contributed to the reduction of neuronal spiking produced by CTX 3C. In this sense, other toxins affecting VGSC such as veratridine induced a sustained membrane depolarization reducing neuronal firing[54] and elicited a GABAergic tonic current in several neuronal preparations.[47] Furthermore, other sodium channel activators such as brevetoxin have been reported to increase GABAergic activity.[55]

The modulation of GABAergic tonic currents represents a mechanism of homeostatic plasticity, allowing a neuron to control its excitability in response to changes in synaptic activity in a short-term time scale.[56] However, other homeostatic mechanisms such as

the maintenance of the balance between excitation and inhibition are essential to maintain neuronal stability[57] and could potentially contribute to the rapid increase in inhibition elicited by CTX 3C in cortical neurons.

In response to changes in activity cortical neurons need to adjust their synaptic strengths up or down to compensate these alterations, a mechanism generally considered to be very slow.[45] However, it has been reported that an acute blockade of synaptic activity (4 hours), induced rapid synaptic scaling mechanisms, very similar to those observed at 24 h, increasing mEPSCs amplitude and the accumulation of AMPA receptors.[48] While changes in the amplitude of mEPSCs and mIPSCs indicate actions on postsynaptic receptors, changes in the frequency of mEPSCs and mIPSCs are indicative of actions on presynaptic neurotransmitter release.[58] Here, the effect of CTX 3C was associated with opposite actions on excitatory and inhibitory postsynaptic receptors as evidenced by a decrease in mEPSC amplitude and an increase in mIPSC amplitude. Moreover, the increase in the frequency of mIPSCs indicates that CTX 3C increased the rate of spontaneous GABA release as mentioned before, while the toxin did not affect the release of glutamate.

Altogether these results indicate that an immediate perturbation of neuronal activity by acute exposure of cortical neurons to CTX 3C might induce rapid disturbances in neuronal homeostasis, as evidenced by the alterations on GABAergic tonic currents and on the shift towards inhibition that CTX 3C induced in the balance between excitation and inhibition. We suggest that these alterations of neuronal synaptic homeostasis probably underlie some of the neurological disturbances observed after ciguatera food poisoning in humans.

Despite the fact that most of the effects of ciguatoxins in humans can be explained by peripheral mechanisms, ciguatera food poisoning also presents with symptoms of central origin that can be linked to the effects observed in this study. For example, the enhancement of the tonic $GABA_A$ conductance leads to altered body temperature and ataxia,[15] both observed in ciguatera.[11,16] Hypothermia, which is common in ciguatera patients,[59] can also be related to a reduction of excitatory synaptic activity.[60] In addition, other symptoms common in ciguatera food poisoning such as fatigue, weakness and depression[61] have been related with central nervous system depression caused by decreased excitatory activity, increased inhibitory activity or both,[17] which is the effect caused by CTX 3C in cortical neurons. On the other hand, an imbalance between excitation and inhibition with specific reduction in the amplitude of mEPSCs has been

observed in cortical neurons of mice suffering from memory impairment and reduced mental capacity[62] also common symptoms in ciguatera.[63] It is also worth mentioning that drugs decreasing glutamate excitation and increasing GABA inhibition produce dizziness, headache or memory alterations as side effects,[64] also typical symptoms in ciguatera.[63] All these data strengthen the hypothesis that a rapid modulation of neurotransmission by CTX3C, specifically by up-regulating phasic and tonic $GABA_A$ inhibition and by decreasing excitatory transmission, could contribute to some of the neurological symptoms observed in ciguatera food poisoning.

It has been reported that CTXs have cellular targets other that TTX-sensitive VGSC,[10, 29,35] however, in our cellular model, 5 nM CTX 3C did not affect voltage gated calcium or potassium currents and did not induce calcium influx from extracellular media or calcium release from intracellular stores.[32] Although, one possible explanation for the effects of CTX 3C on synaptic transmission could be the activation of TTX-resistant sodium channels by CTX 3C as it has been reported in other cellular models,[29,33] these channels are mainly expressed in nociceptive sensory neurons[65] and it seems unlikely that the effect of CTX 3C in cortical neurons were mediated by these channels, since 0.5 μM TTX completely blocked sodium currents in cortical neurons.[66]

Other cellular mechanisms such as the activation of transient receptor potential channels could potentially contribute to the neurological effects of ciguatoxins, since these channels are involved in the symptomatology of ciguatera[11] and in synaptic plasticity.[67]

In the present study while the effect of CTX 3C on membrane depolarization was mediated by VGSC since it was blocked by TTX, the modulatory effects of CTX 3C on miniature excitatory and inhibitory postsynaptic currents (recorded in the presence of TTX) and on the GABAergic tonic current were independent of VGSC activation and are suggested to be related with an increase in ambient GABA levels elicited in the presence of the toxin.

Overall, our results indicate that CTX 3C potentiates inhibition in cortical neurons both by an increase in tonic and phasic GABAergic inhibition and through a decrease in excitatory postsynaptic activity. The novel finding that an acute exposure of cortical neurons to CTX 3C induces an immediate modification of excitatory and inhibitory transmission constitutes the first approach that evaluates the effect of a neurotoxin activating sodium channels on neuronal homeostasis. Although the cellular mechanisms involved in the modification of synaptic strength by CTX 3C remain to be determined

the results presented here provide new hypothesis for potential neuronal mechanisms that could be involved in the physiopathology of ciguatera.

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Supporting Information

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

Figure S1. Tonic current induced by GABA in cortical neurons. Representative recording showing the effect of an acute administration of 500 μM GABA on I_{hold}.

Figure S2. Pharmacological isolation of mIPSCs. A, representative trace of spontaneous postsynaptic currents (sPSCs) in control conditions measured at -80 mV. This cell received some large amplitude, presumably action potential-dependent synaptic inputs**. B,** mIPSCs were isolated by the addition of TTX + APV + CNQX. **C,** GABAergic mIPSCs were confirmed by the addition of BIC.

Figure S3. Pharmacological isolation of mEPSCs. A, representative trace of spontaneous postsynaptic currents (sPSCs) in control conditions measured at -80 mV. **B,** mEPSCs were isolated by the addition of TTX + BIC. **C,** Glutamatergic mEPSCs were confirmed by the addition of APV and CNQX.

Abbreviations

CTX, ciguatoxin; VGSC, voltage-gated sodium channels; TTX, tetrodotoxin; mIPSCs, miniature postsynaptic inhibitory currents; mEPSCs, miniature postsynaptic excitatory

currents; PbTx, brevetoxin; NMDA, N-methyl-D-aspartate receptor; BIC, bicuculline; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; APV, D(-)-2-amino-5-phosphonopentanoic acid; $V_{m,}$ membrane potential; sPSC spontaneous postsynaptic current; I_{hold}, holding current; I_{Na}, sodium current amplitude; TTX-R, tetrodotoxin resistant.

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Figure legends

Figure 1. Concentration-dependent effects of CTX 3C on sodium currents (I_{Na}). A, I-V relationship for the effect of 1 nM and 5 nM CTX 3C on I_{Na} in cortical neurons. **B**, pooled results for the concentration dependent effects of CTX 3C on I_{Na} activation. Note that both 1 nM and 5 nM CTX 3C shifted the activation of I_{Na} to more hyperpolarized potentials. **C,** representative peak sodium currents in the absence and presence of 5 nM CTX 3C. **D,** pooled results of I_{Na} measured at -10 mV in the absence and presence of 5 nM CTX 3C. The number of cells tested is indicated in parentheses. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

Figure 2. CTX 3C decreases spontaneous neuronal spiking in a concentration-dependent manner. A, example of voltage traces of a neuron showing spontaneous

firing activity when V_m was permitted to fluctuate freely. Addition of 1 nM CTX 3C did not modify the firing activity. **B**, example of voltage traces of a neuron showing spontaneous firing activity when V_m was permitted to fluctuate freely. Addition of 5 nM CTX 3C (arrow) depolarized the membrane potential and abolished the firing activity. **C,** pooled results of the frequency (left panel) and amplitude (right panel) of the spikes in the absence (control) and in presence of 1 nM CTX 3C (n = 4). **D,** pooled results of the frequency (left) and amplitude (right) of the spikes in the absence (control) and in presence of 5 nM CTX 3C (n = 10). The number of cells tested is indicated in parentheses. **p < 0.01, ***p < 0.005.

Figure 3. CTX 3C produces neuronal depolarization and reduces neuronal spiking. **A**, example of a voltage and a current trace of a neuron showing spontaneous firing activity when V_m was clamped at -55 mV before and after the addition of 1 nM CTX 3C. Amplified recordings from the same neuron, before and after addition of the toxin, are shown in the lower panels. **B,** example of a voltage and a current trace of a neuron showing spontaneous firing activity when V_m was clamped at -55 mV before and after the addition of 5 nM CTX 3C indicating that the current necessary to maintain V_m at -55 mV was enhanced after bath application of 5 nM CTX 3C. Amplified recordings from the same neuron, before and after addition of the toxin, are shown in the lower panels. **C,** pooled results showing spike frequency (left) and amplitude (right) in the absence (control) and in presence of 1 nM CTX 3C when V_m was held at -55 mV. **D,** pooled results showing spike frequency (left) and amplitude (right) in the absence (control) and presence of 5 nM CTX 3C when V_m was held at -55 mV. **E**, histogram showing the mean membrane potential before and after addition of 1 nM and 5 nM CTX 3C (left and right panel, respectively). **F**, the depolarizing effect of 5 nM CTX 3C was abolished in the presence of TTX. The number of cells tested is indicated in parentheses. **p < 0.01, ***p < 0.005.

Figure 4. CTX 3C effects on I_{hold} and spontaneous postsynaptic currents in cortical neurons. CTX 3C activated a tonic inward current. **A**, representative trace of the effect of 5 nM CTX 3C on the holding current (I_{hold}) when V_m was clamped at -80 mV. **B**, Quantitative analysis showing de effect of CTX 3C, at 1 and 5 nM in I_{hold}. **C**, detailed traces of mixed sPSCs recorded before (control) and after 1 nM CTX 3C (upper panel) and 5 nM CTX 3C (lower panel) bath application at a holding potential of -80 mV. **D**,

distribution of the sPSCs from 3 neurons in control conditions and in the presence of 1 nM CTX 3C (101 events per condition were pooled) and distribution of the sPSCs from 7 neurons in control conditions and in the presence of 5 nM CTX 3C (381 events per condition were pooled). Note the shift to the right in sPSCs amplitude in the presence of 5 nM CTX 3C but not at the lower concentration of the toxin. **E**, plotted average values of sPSCs amplitude in the absence (control) and presence of 1 and 5 nM CTX 3C. **F**, plotted values of sPSCs area in the absence (control) and in the presence of 1 and 5 nM CTX 3C. *$p < 0.05$, ***$p < 0.001$. The number of the cells analyzed is shown in parenthesis.

Figure 5. The tonic current induced by 5 nM CTX 3C was mainly GABAergic. A, Quantitative analysis of I_{hold} values in control conditions and in the presence of 5 nM CTX 3C alone or after the consecutive addition of TTX and BIC. Note that the shift on I_{hold} produced by CTX 3C was not reversed by TTX but it was completely blocked by BIC. **B,** a representative recording of the effect of 5 nM CTX 3C on the holding current. **C,** values of I_{hold} (upper panel) and V_m (lower panel) in a representative cell indicating the relationship between the shifts in I_{hold} and V_m elicited by 5 nM CTX 3C. **D,** quantitative analysis of I_{hold} values obtained after bath application of 5 nM CTX 3C in the simultaneous presence of TTX and BIC (upper panel) and representative recording showing that in the presence of TTX and BIC I_{hold} was not modified by 5 nM CTX 3C (lower panel). **$p < 0.01$, ***$p < 0.001$. The number of the cells analyzed is shown in parenthesis. Dashed lines indicate baseline I_{hold}.

Figure 6. CTX 3C potentiates GABAergic activity. A, an example of mIPSCs (in the presence of TTX, APV and CNQX) recorded as inward currents at a holding potential of -80 mV. The arrow indicates the administration of 5 nM CTX 3C. Dashed line shows baseline holding current. **B**, detailed traces of mIPSCs from the cell in A recorded before (left) and after bath application of 5 nM CTX 3C (right). Distribution of the mIPSCs from the same neuron one minute before (left) and 4 minutes after administration of 5 nM CTX 3C are shown (25 events per condition were pooled). **C**, histogram showing the distribution of mIPSCs pooled from 5 neurons and recorded one minute before (control) and 4 minutes after bath application of 5 nM CTX 3C (130 events per condition were pooled). **D**, Mean normalized values of the amplitude, frequency and area of mIPSCs in the absence (control) and presence of 5 nM CTX 3C

(n = 4, *p < 0.05). **E**, average traces of mIPSCs in the absence (control) and presence of CTX 3C obtained from 4 cells.

Figure 7. CTX 3C reduces mEPSCs amplitude. A, An example of mEPSCs (recorded in the presence of TTX and BIC) that appear as inward currents at a holding potential of -80 mV. The arrow indicates bath application of 5 nM CTX 3C. **B**, detailed traces of mEPSCs from the cell in A, recorded before (left) and after bath application of 5 nM CTX 3C (right). The distribution of the mEPSCs recorded from the same neuron one minute before (left) and 4 minutes after addition of 5 nM CTX 3C is shown below the traces (50 events per condition were pooled). **C**, histogram showing the distribution of mEPSCs obtained from 4 neurons one minute before (control) and 4 minutes after bath application of 5 nM CTX 3C (150 events per condition were pooled). **D**, quantitative analysis of the normalized values of the amplitude, frequency and area of mEPSCs in the absence (control) and in the presence of 5 nM CTX 3C (n = 9, **p < 0.01). **E**, average traces of mEPSCs in the absence (control) and presence of CTX 3C obtained from 9 cells (upper panel) and their average CTX 3C-sensitive current obtained by subtracting the remaining current after application of CTX 3C from the control mEPSC current (recorded in the presence of TTX and BIC) as averaged from 9 independent cells (lower panel).

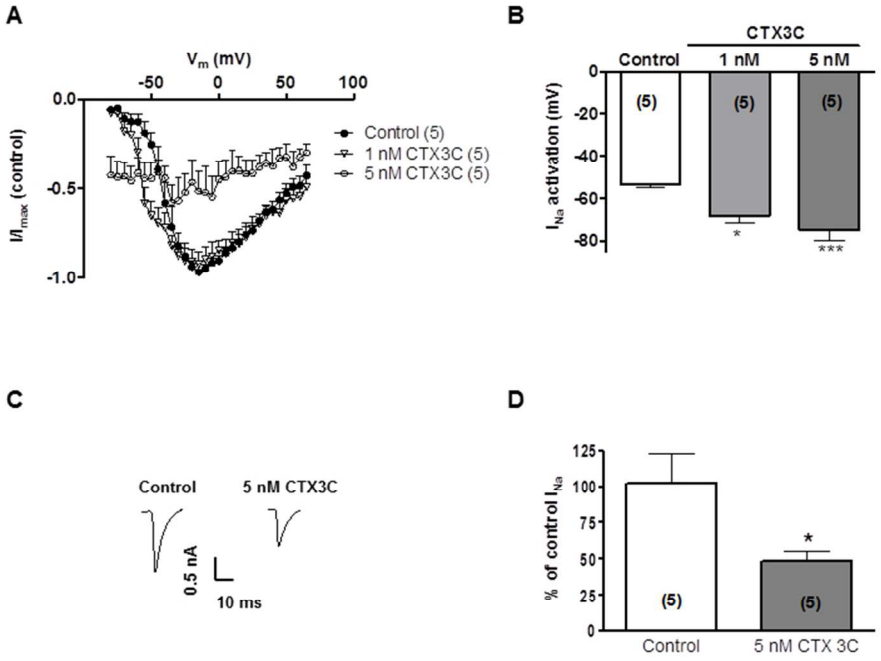

Figure 1

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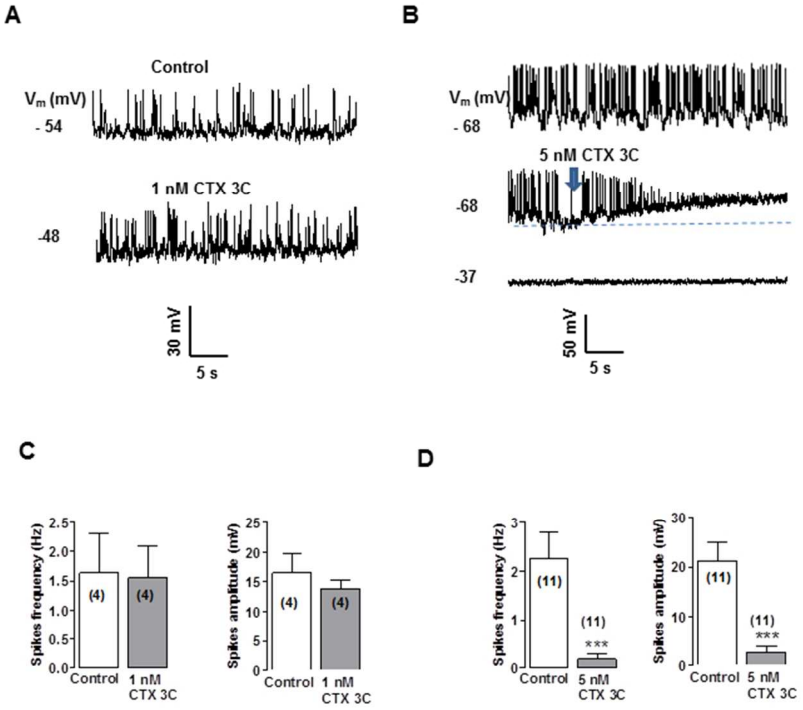

Figure 2

190x254mm (96 x 96 DPI)

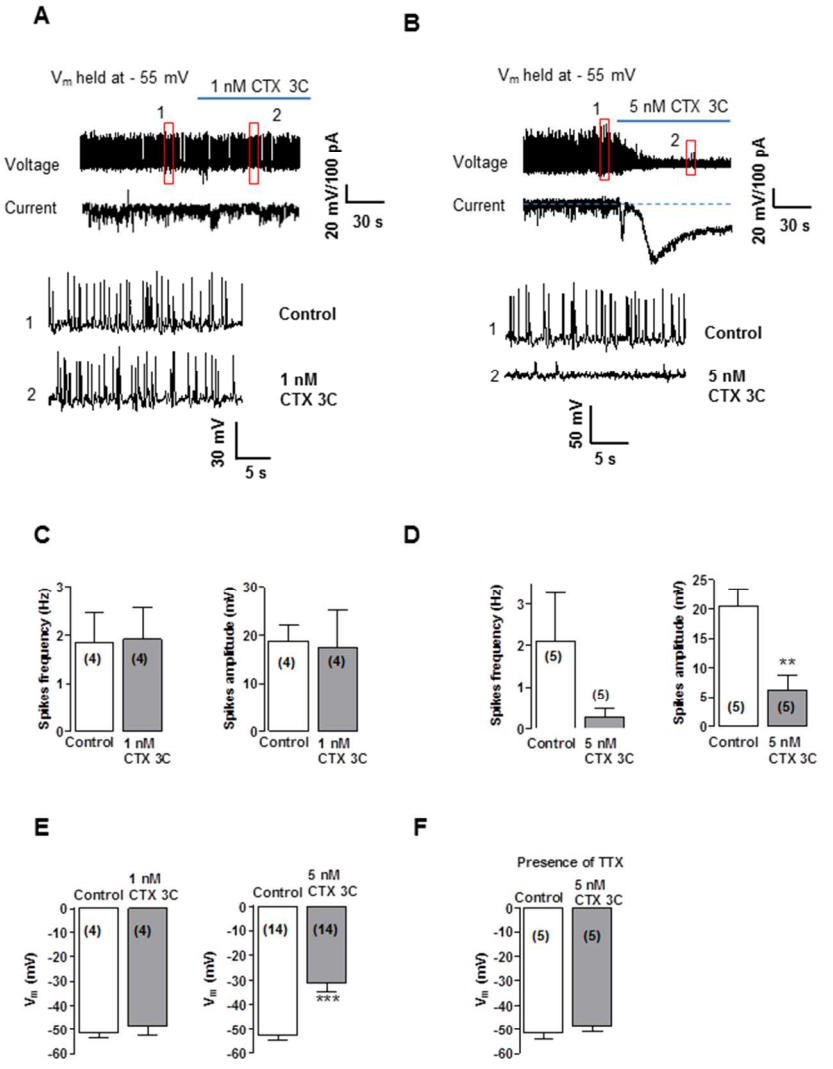

Figure 3

190x254mm (96 x 96 DPI)

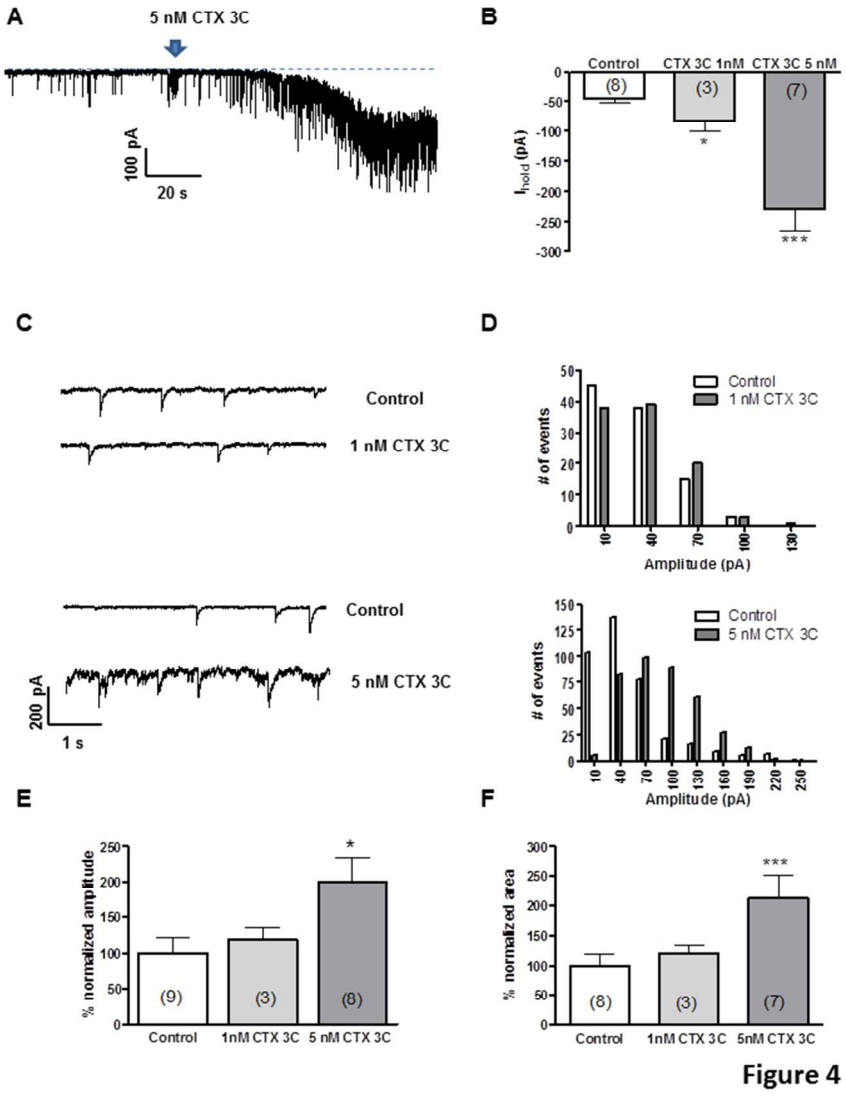

Figure 4

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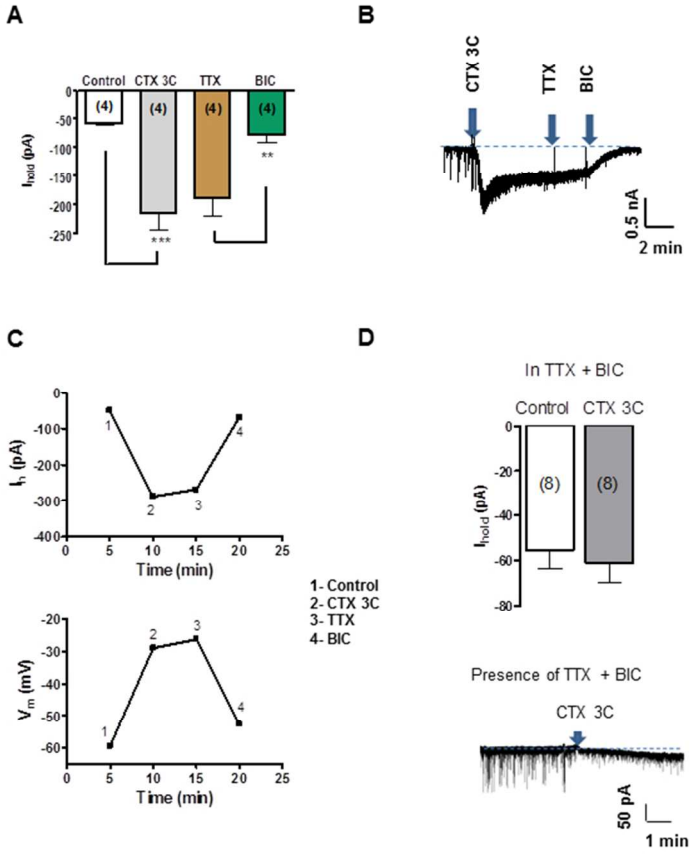

Figure 5

190x254mm (96 x 96 DPI)

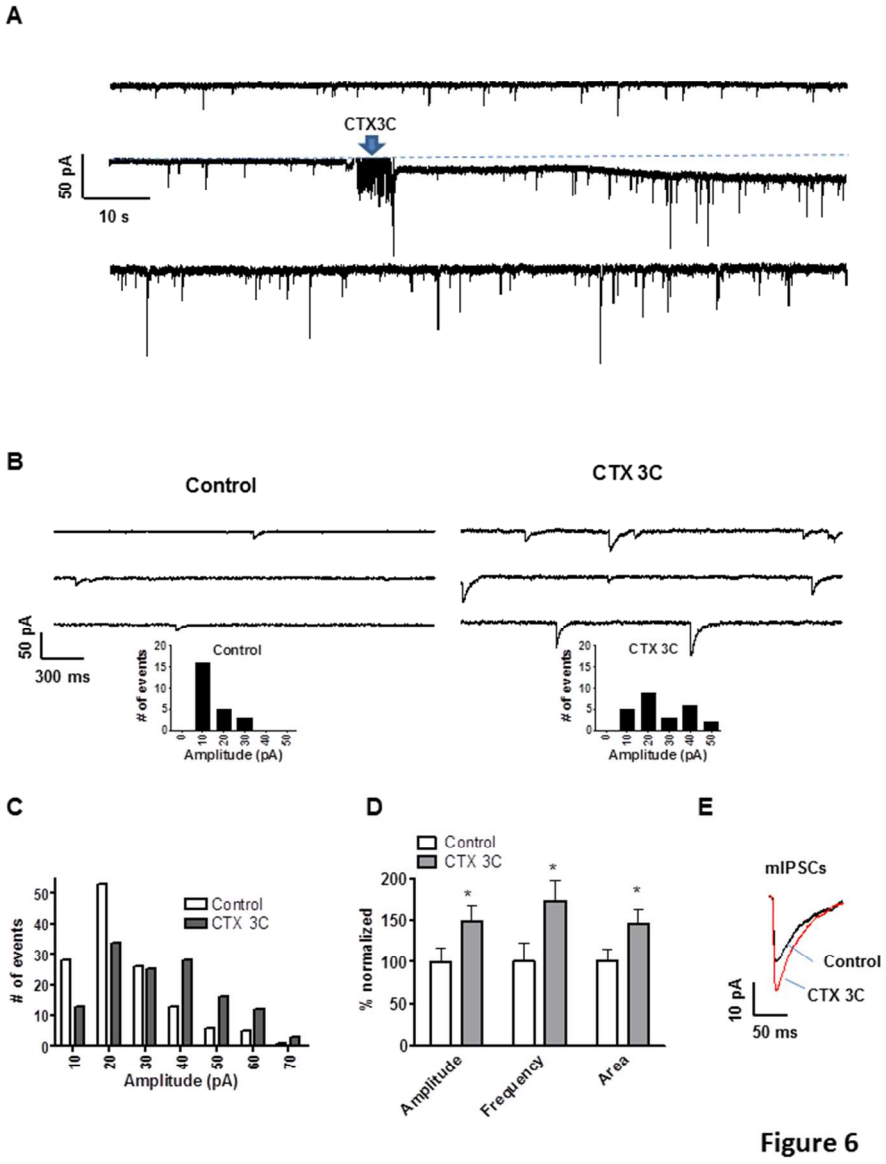

Figure 6

190x254mm (96 x 96 DPI)

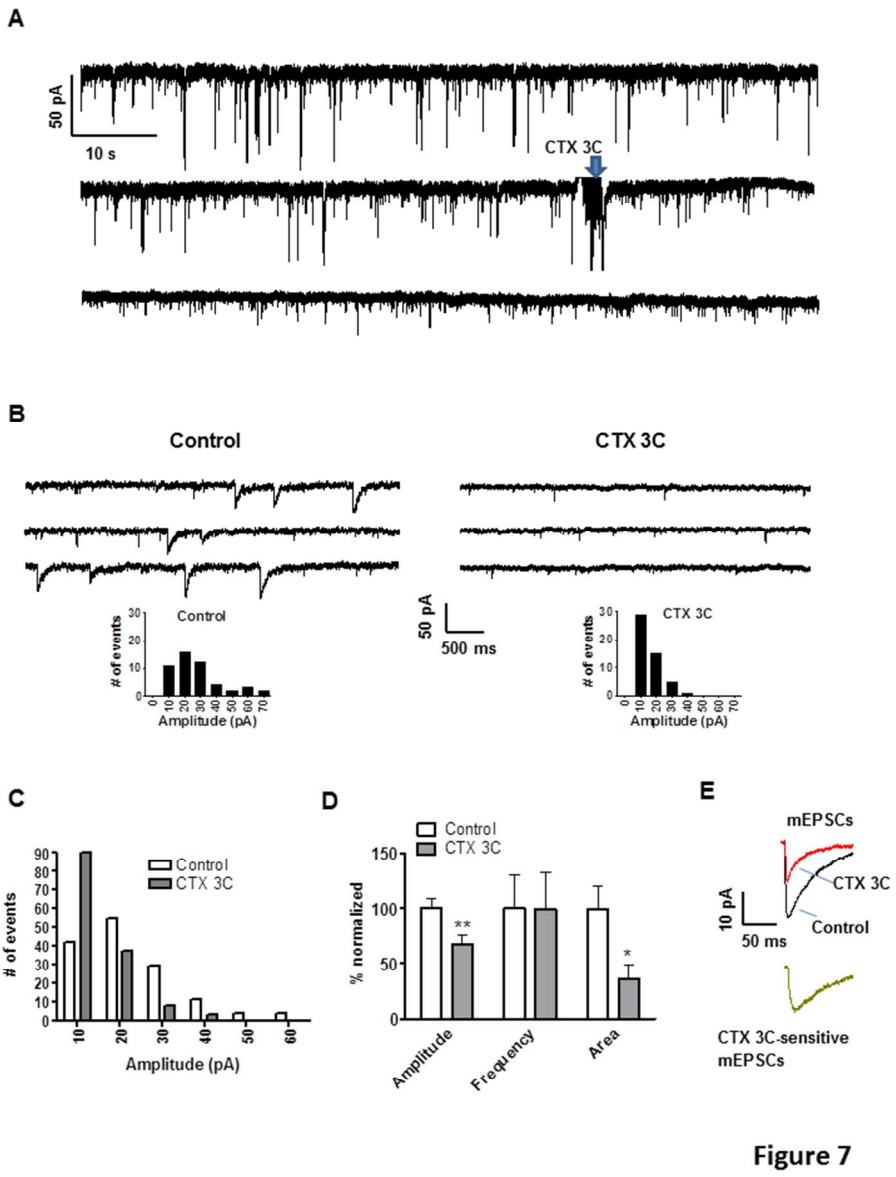

Figure 7

190x254mm (96 x 96 DPI)