

4,5-Dichloro-2-octyl-4-isothiazolin-3-one (DCOIT) modifies synaptic transmission in  
hippocampal CA3 neurons of rats

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## 1. Introduction

In response to the banning of organotin antifoulants, such as tributyltin and triphenyltin, chemical companies have actively developed alternative biocides (Qian et al., 2013). 4,5-Dichloro-2-octyl-4-isothiazolin-3-one (DCOIT) is supposed to be an environmentally acceptable alternative to organotin antifoulants (Jacobson and Willingham, 2000). DCOIT is the active ingredient in a series of biocide formulations marketed by the Dow Chemical Company and its global affiliates (The Dow Chemical Company, 2012). DCOIT was found in sediments of port, harbor, and coastal areas in Asian countries (Harino et al., 2007; Tsunemasa and Yamazaki, 2014; Kim et al., 2015a,b; Mochida et al., 2015). The octanol-water partition coefficient of DCOIT is reported to be 4.5 (Shade et al., 1993) or 2.85 (Willingham and Jacobson, 1996). Thus, since there is a possibility that DCOIT accumulates in organisms, this compound may have impacts on ecosystems (Guardiola et al., 2012). The toxicity of DCOIT in wild animals is not reported. However, DCOIT is found to possess adverse actions in *in vivo* studies (Ito et al., 2013; Chen et al., 2014a,b, 2015, 2016). DCOIT induced apoptosis of testicular germ cells in *mummichog* (Ito et al., 2013). In marine *Medaka*, DCOIT altered oxidative stress and neurotransmission, caused endocrine disruption, and induced proteomic changes in brain and liver tissues (Chen et al., 2014a,b, 2015, 2016). DCOIT significantly inhibited acetylcholinesterase activity in the brain of marine *Medaka* (Chen et al., 2014a). The inhibition of acetylcholinesterase produces effects reminiscent of neurotoxicity, as described for some insecticides (Veronesi et al., 1990; Russom et al., 2014). Therefore, the aim of this study is to examine whether DCOIT has adverse actions on neuronal transmission, especially in mammalian CNS. Synaptic transmission is one of main targets for pathological and toxicological studies because it is the most vulnerable step in neuronal transmission (or signaling). Parkinson's disease, schizophrenia, dementia, and depression are related to malfunction of synaptic transmission. Therefore, this study possesses toxicological and pathological (probably biological) implications. Methyl mercury (MetHg) is one of

environmental pollutants. This compound is known to cause ‘Minamata Disease’. MetHg acts on neuronal functions including synaptic transmission in various brain regions. Thus, it is important to conduct the experiment to reveal the effects of environmental pollutants on the neuronal transmission. In this study, we examined the effects of DCOIT on synaptic transmission using a ‘synaptic bouton’ preparation (Akaike et al., 2002; Akaike and Moorhouse, 2003) isolated from hippocampus of rat brain. Hippocampal neurons are a sensitive, well-established assay for synaptic transmission function. Here, we describe the actions of DCOIT on synaptic transmission mediated by GABA and glutamate in rat brain. These actions are mediated mainly via the mobilization of intracellular  $\text{Ca}^{2+}$ . The results suggest that DCOIT at its present environmentally relevant concentrations in sea sediments may be neurotoxic in wild mammals.

## 2. Materials and Methods

### 2.1. Cell preparation – ‘synaptic bouton’ preparation

The use of experimental animals was approved by the Ethics Committee of Kumamoto Kinoh Hospital. All experiments were performed in accordance with the Guiding Principles for Care and Use of Animals in the Field of Physiological Sciences of The Physiological Society of Japan.

Details of the “synaptic bouton” preparation were described previously (Akaike et al., 2002; Akaike and Moorhouse, 2003). Briefly, Wistar rats (11–23 days old, either sex) were decapitated under pentobarbital anesthesia. The brain was removed and immersed in ice-cold oxygenated incubation medium. The ionic composition of the incubation medium was 124 mM NaCl, 5 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 24 mM  $\text{NaHCO}_3$ , 2.4 mM  $\text{CaCl}_2$ , 1.3 mM  $\text{MgSO}_4$  and 10 mM glucose. The medium was saturated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  in order to adjust the pH to 7.45.

Hippocampal slices (400- $\mu\text{m}$  thick) were prepared using a vibrating microtome (VR

1200S; Leica, Nussloch, Germany) and then incubated in the medium at room temperature (21–24 °C) for at least one hour before mechanical dissociation using a fire-polished glass pipette coupled to a vibration device (S1-10 cell isolator; K.T. Labs, Tokyo). The tip of the glass pipette was placed on the surface of the slice in the hippocampal CA3 region and was vibrated horizontally (0.2–2.0 mm displacement) at 50 Hz. After dissociation, the mechanically dissociated neurons were left to settle and adhere to the bottom of the dish for at least 15 minutes.

## 2.2. Electrophysiological measurements

All recordings were obtained from the ‘synaptic bouton’ preparation of CA3 pyramidal neurons using conventional whole-cell patch-clamp recordings in voltage-clamp mode. Tables 1 and 2 show the ionic compositions of the solutions used for the current recordings. Glutamatergic evoked, spontaneous, and miniature excitatory postsynaptic currents (eEPSCs, sEPSCs, mEPSCs), and glutamate receptor-mediated currents ( $I_{\text{Glu}}$ ) were recorded at a holding potential ( $V_{\text{H}}$ ) of  $-65$  mV. GABAergic evoked, spontaneous, and miniature inhibitory postsynaptic currents (eIPSCs, sIPSCs, mIPSCs) and GABA<sub>A</sub> receptor-mediated currents ( $I_{\text{GABA}}$ ) were recorded at a  $V_{\text{H}}$  of 0 mV. Voltage-dependent Na<sup>+</sup> channel currents ( $I_{\text{Na}}$ ) and Ba<sup>2+</sup>-permeable high-threshold Ca<sup>2+</sup> channel currents ( $I_{\text{Ba}}$ ) were recorded  $V_{\text{HS}}$  of  $-70$  mV and  $-60$  mV, respectively (Multiclamp 700B; Molecular Devices, Sunnyvale, CA) (Wakita et al., 2012). All experiments were performed at room temperature (21–24 °C).

The resistances of the recording pipettes filled with the internal solution were 3–6 MΩ. Neuronal responses were continuously monitored on a computer display and an oscilloscope (DCS-7040; Kenwood, Tokyo, Japan). All membrane currents were acquired with 20 kHz sampling rate, filtered at 3 kHz using a low-pass filter (Multifunction Filter 3611; NF Co., Tokyo, Japan) and stored on a computer using pCLAMP 10.2 (Axon Instruments, CA, USA).

(Tables 1 and 2 near here)

Experiments were performed with wide range (0.03–10 μM) of DCOIT concentration.

One may argue the possibility that DCOIT decreases cell viability. In electrophysiological studies, it is impossible to record electrical response from dead cells or the cells with compromised membranes. Thus, electrophysiological recording reveals whether recorded cell is viable.

### 2.3. Paired-pulse focal electrical stimulation of single boutons using glass pipettes

Focal electric stimuli can be employed to activate a single nerve terminal in order to measure eIPSCs and eEPSCs resulting from a single presynaptic nerve ending rather than a fused event from multiple boutons. This technique offers a unique evaluation of how drugs act on pre- and post-synaptic transmission mechanisms at the single synapse level. Focal electrical stimulation of a single bouton adherent to mechanically dissociated hippocampal CA3 neurons has been described previously (Akaike et al., 2002; Akaike and Moorhouse, 2003; Wakita et al., 2012). Focal activation was achieved using a bipolar stimulating pipette (theta glass) for both current delivery and the return path in a compact format. Stimulus shocks were delivered for 100  $\mu$ s at intensities of 0.06–0.1 mA with inter-stimulus intervals of 40 ms for eIPSCs and 20 ms for eEPSCs (Wakita et al., 2013). The stimulating electrode was carefully moved along the surface membrane but did not directly touch the membrane of a bouton. Periodic shocks were delivered while monitoring whole-cell currents until eIPSCs or eEPSCs appeared. These synaptic events appeared in an all-or-none fashion over a limited range of positions, which indicates that effective stimulation requires the pipette to be positioned just above the bouton (Akaike et al., 2002; Akaike and Moorhouse, 2003). Membrane resistance was not changed during the application of DCOIT because the base-line of current was not changed.

### 2.4. Drugs and Application

DCOIT was distributed by Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The purity of DCOIT was >99.8%. Reagents to prepare the solutions described below were obtained from Wako Pure Chemicals (Osaka, Japan). Other reagents, such as tetraethylammonium chloride (TEA-Cl), CsCl, Cs-methanesulfonate, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

(HEPES), tetrodotoxin (TTX), ethyleneglycol-bis-( $\alpha$ -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 2-((2,6-dimethylphenyl)amino)-N,N,N-triethyl-2-oxoethanaminium (QX-314), and adenosine 5'-triphosphate magnesium salt (ATP-Mg) were obtained from Sigma Chemicals (St. Louis, MO, USA). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) and D-(-)-2-amino-5-phosphonovaleric acid (D-AP5) were obtained from Tocris Cookson (Ellisville, MO, USA). All test solutions containing drugs were applied using a 'Y-tube system,' which allowed solution exchange around the cells within about 20 ms (Murase et al., 1990).

## 2.5. Data analysis

GABAergic eIPSCs and glutamatergic eEPSCs evoked by paired-pulse focal electrical stimuli were counted and analyzed in pre-set epochs before, during, and after each test condition using pCLAMP 10.2 (Axon Instruments). The amplitudes, failure rates (Rfs), and paired-pulse ratios (PPRs) of eIPSCs and eEPSCs were analyzed using pCLAMP 10.2. PPRs were calculated by dividing the mean peak amplitude of  $P_2$  by the mean peak amplitude of  $P_1$ . The effects of DCOIT on the current amplitudes, Rfs, and PPRs for eIPSCs and eEPSCs were normalized as relative changes with respect to their respective controls. Mean amplitude was calculated from all current responses including current amplitude = 0 (full failure).

To examine the effects of DCOIT on ionotropic inward currents induced by bath perfusions of GABA ( $I_{GABA}$ ) and glutamate ( $I_{Glu}$ ), 3  $\mu$ M GABA and 10  $\mu$ M glutamate were applied. These drug concentrations elicited little desensitization. The concentration-dependent effects of DCOIT were quantified by normalizing the peak current amplitudes in the presence of DCOIT to those in the absence of DCOIT. The relative  $I_{Na}$  and  $I_{Ba}$  responses with various concentrations of DCOIT were similarly normalized to the control peak currents in the standard solution without DCOIT.

In each experiment, sIPSCs, sEPSCs, mIPSCs, and mEPSCs were counted and analyzed in pre-set epochs before, during, and after each test condition using the MiniAnalysis Program (Synaptosoft, NJ, USA). Briefly, synaptic events were initially screened automatically using an

amplitude threshold of 10 pA and then visually accepted or rejected based upon their 10–90% rise and 90–37% decay times. The frequency, amplitude, and decay times (90–37%) of synaptic events during the control period (3 minutes) were averaged, and the frequencies, amplitudes, and decay times of all events during drug application were normalized to these control values.

All Data were analyzed using Origin Pro 7.5 software (OriginLab Corporation, Northampton, MA, USA). Data are reported as means  $\pm$  S.E.M. of normalized values. Data were tested using one-way ANOVA followed by a post hoc Bonferroni test for multiple comparisons, using absolute values rather than normalized ones. Two-tailed P values of less than 0.05 were considered statistically significant.

### 3. Results

#### 3.1. Effects of DCOIT on postsynaptic currents evoked by focal electrical stimulation

Synaptic boutons projecting to single CA3 neurons were stimulated by focal electrical stimulation (Akaike and Moorhouse, 2003; Akaike et al., 2002). The upper insets in Figs. 1A and 1B show typical outwardly-directed, fast eIPSCs in response to paired-pulse stimulation (40-ms interval) with and without 0.3  $\mu$ M DCOIT and 10  $\mu$ M DCOIT, respectively. DCOIT at 0.3  $\mu$ M increased the amplitude of the current elicited by the P<sub>1</sub> pulse (P<sub>1</sub> Amp), while DCOIT at 10  $\mu$ M greatly diminished the P<sub>1</sub> Amp. There was no recovery from the response to DCOIT after the removal of DCOIT surrounding the preparation (Figs. 1A and B).

The upper insets in Figs. 1C and 1D show typical inwardly-directed, fast eEPSCs in response to paired-pulse stimulation (20-ms interval) with and without 0.3  $\mu$ M DCOIT or 10  $\mu$ M DCOIT, respectively. DCOIT at 0.3  $\mu$ M increased the P<sub>1</sub> Amp. There was recovery from the response to DCOIT after washing out. DCOIT at 10  $\mu$ M significantly decreased the P<sub>1</sub> Amp. In this case, there was no recovery from the response to DCOIT after its removal (Figs. 1C and 1D).

(Figure 1 near here)

The concentration-dependent changes in the  $P_1$  Amps of eIPSCs and eEPSCs elicited by DCOIT are summarized in Fig. 2A. Concentrations of DCOIT ranging from 0.03  $\mu\text{M}$  to 1  $\mu\text{M}$  increased the  $P_1$  Amp, while there were decreases in response to 3–10  $\mu\text{M}$  DCOIT. DCOIT at 0.03  $\mu\text{M}$  decreased the value of the  $P_1$  Rf in eEPSCs, but not eIPSCs. The Rf values of both evoked currents were reduced by 0.1–0.3  $\mu\text{M}$  DCOIT, while 3–10  $\mu\text{M}$  DCOIT increased these values (Fig. 2B). Similar tendencies were observed for DCOIT-induced changes in relative PPR (Fig. 2C). Thus, DCOIT biphasically modulates synaptic transmissions.

(Figure 2 near here)

### 3.2. Effect of DCOIT on $I_{\text{GABA}}$ and $I_{\text{Glu}}$ elicited by exogenous applications of GABA and glutamate

There is a possibility that DCOIT directly affects the synaptic currents evoked by focal electrical stimulation, resulting in the changes in the  $P_1$  Amp. However, this possibility is unlikely, as DCOIT at concentrations of 0.03–3  $\mu\text{M}$  did not change the amplitudes of  $I_{\text{GABA}}$  and  $I_{\text{Glu}}$  elicited by exogenous applications of GABA and glutamate (supplemental Fig. 1). Our results thus suggest that the effects of DCOIT on eIPSCs and eEPSCs resulted from its actions at presynaptic sites.

### 3.3. Effects of DCOIT on sIPSCs and sEPSCs

As shown in Figs. 3A and 3B, DCOIT at 10  $\mu\text{M}$  significantly increases the numbers of both sIPSC and sEPSC events recorded during a period of 20 seconds. The increased numbers of events gradually decreased after the removal of DCOIT. The effects of DCOIT at 0.03–10  $\mu\text{M}$  on sIPSCs and sEPSCs are summarized in Fig. 3C. DCOIT at concentrations of 1  $\mu\text{M}$  or higher (up to 10  $\mu\text{M}$ ) significantly increases the frequency of sIPSCs in a concentration-dependent manner (Fig. 3C). Similarly, the frequency of sEPSCs was significantly increased in response to DCOIT concentrations of 0.3–10  $\mu\text{M}$ . The amplitudes of sIPSCs were significantly increased by 3–10  $\mu\text{M}$  DCOIT. The amplitudes of sEPSCs were only increased by 10  $\mu\text{M}$



DCOIT. The decay times of sIPSCs and eEPSCs were not affected by DCOIT at concentrations of up to 10  $\mu\text{M}$ . Thus, it seems that DCOIT acts at presynaptic sites.

(Figure 3 near here)

#### 3.4. Effects of DCOIT on mIPSCs and mEPSCs

TTX was applied under external  $\text{Ca}^{2+}$ -free conditions and mIPSCs and mEPSCs were recorded (Figs. 4A and 4B). In the presence of TTX under external  $\text{Ca}^{2+}$ -free conditions, the frequency of events was reduced. The application of 10  $\mu\text{M}$  DCOIT time-dependently and markedly increased the frequencies of both mIPSCs and mEPSCs. The increases in these frequencies in response to DCOIT were statistically significant (Fig. 4C). The amplitudes of mIPSCs were reduced in the presence of TTX under external  $\text{Ca}^{2+}$ -free conditions. This reduction was statistically significant. Successive applications of 10  $\mu\text{M}$  DCOIT also increased the amplitude by a small amount (Fig. 4C). A similar tendency was observed for mEPSCs. However, the changes in these amplitudes were not statistically significant.

(Figure 4 near here)

#### 3.5. Effects of repetitive DCOIT applications on mEPSCs

As shown in Figs. 2 and 3, the frequency of EPSCs was more sensitive to DCOIT than that of IPSCs. Further studies were conducted on mEPSCs. Voltage-dependent  $\text{Na}^+$  and  $\text{Ca}^{2+}$  channels are not involved in responses to TTX under external  $\text{Ca}^{2+}$ -free conditions. Therefore, it is hypothesized that intracellular  $\text{Ca}^{2+}$  released from  $\text{Ca}^{2+}$  stores contributes to the increase in mEPSC frequency. DCOIT at 10  $\mu\text{M}$  was repetitively applied in the presence of TTX under external  $\text{Ca}^{2+}$ -free condition. When 10  $\mu\text{M}$  DCOIT was intermittently applied, the increase in mEPSC frequency decreased in a step-wise manner by the second and third applications of 10  $\mu\text{M}$  DCOIT (Figs. 5A and 5Ba). However, the amplitude was not affected at all (Fig. 5Bb).

(Figure 5 near here)

### 3.6. Effects of DCOIT on voltage-dependent Na<sup>+</sup> and Ca<sup>2+</sup> channel currents

Fig. 6A shows the  $I_{Na}$  recording traces without and with DCOIT. DCOIT at 3–10  $\mu$ M slowed the decay phase of the  $I_{Na}$  without inducing an apparent change in current amplitude. DCOIT at concentrations ranging from 0.1  $\mu$ M to 10  $\mu$ M increased the decay time in a concentration-dependent manner. While the amplitude of the  $I_{Na}$  was hardly affected by DCOIT, increases in decay time of the  $I_{Na}$  due to DCOIT were statistically significant in the concentration range of 1–10  $\mu$ M (Fig. 6B). On the other hand, DCOIT at 0.03–10  $\mu$ M had no effects on  $I_{Ba}$  (supplemental Fig. 2).

(Figure 6 near here)

## 4. Discussion

### 4.1. DCOIT-induced changes in synaptic transmission

From the results of Figs. 1–3, DCOIT is considered to act on presynaptic nerve terminals, resulting in the facilitation of both spontaneous and action potential-dependent transmitter release. DCOIT-induced facilitation of transmitter release does not depend on Ca<sup>2+</sup> influx across the plasma membrane (Fig. 4). The facilitation of transmitter release in the presence of DCOIT results from Ca<sup>2+</sup> release from intracellular Ca<sup>2+</sup> storage sites (Fig. 5). The present results are similar to those reported in the studies on other pollutants, such as MeHg (Juang and Yonemura, 1975), tri-n-butyltin (Kishimoto et al., 2001), and triphenyltin (Oyama et al., 1992; Wakita et al., 2015). Therefore, it may be likely that the neurotoxic actions of DCOIT are partly similar to those of organometals.

The increase in the frequency of mEPSCs was counteracted by repetitive applications of DCOIT in the Ca<sup>2+</sup>-free solution containing TTX (Fig. 5). Thus, the repetitive DCOIT applications deplete the Ca<sup>2+</sup> in intracellular Ca<sup>2+</sup> stores in nerve endings. Such Ca<sup>2+</sup> depletion may also be involved in the inhibition of action potential-dependent transmitter release at high DCOIT concentration because of following reasons. The Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release (CICR)

(Verkhatsky and Shmigol, 1996) is activated by  $\text{Ca}^{2+}$  influx through voltage-dependent  $\text{Ca}^{2+}$  channels that were not inhibited by DCOIT. Therefore, there is another possibility that DCOIT inhibit CICR via a  $\text{Ca}^{2+}$  depletion of calcium stores, resulting in the depression of action potential-dependent transmitter release. One may argue a following possibility. The distribution of voltage-dependent  $\text{Ca}^{2+}$  channel subtypes in postsynaptic membranes might be different from that in presynaptic membranes. However, our previous studies showed that the difference of  $\text{Ca}^{2+}$  channel subtypes between pre- and postsynaptic membrane are a little. On this matter, we investigated electrophysiologically what kinds of  $\text{Ca}^{2+}$  channel subtypes exist in presynaptic nerve terminals (Murakami et al., 2002; Shin et al., unpublished observation).

In previous studies (Yamamoto et al., 2011, Wakita et al., 2012), the large depolarization of presynaptic nerve terminals facilitates spontaneous transmitter release, but inhibits action potential-dependent release by blockage of  $\text{Na}^+$  channels. At high concentration DCOIT induced a slight increase in  $I_{\text{Na}}$  decay time (Fig. 6), possibly resulting in prolonged action potential. Therefore, one may argue a possibility that the prolongation of action potential by DCOIT sustainably depolarizes the presynaptic terminals, resulting in the depression of action potential-dependent transmitter release. However, it seems to be unlikely because the slight prolongation of action potential by DCOIT is not enough to consistently depolarize the presynaptic membranes.

The effects of DCOIT were about 10 to 30 times greater on eIPSCs and eEPSCs than on spontaneous currents. In addition, the former was drained much faster than the latter. It is well-known that the release of both GABA and glutamate at many synapses is facilitated by a presynaptic cAMP increase, which activates PKA and the phosphorylation of presynaptic proteins involved in vesicle cycling and/or exocytosis (Chavis et al., 1998; Turner et al., 1999). Other reports show the cAMP modulates release via PKA-independent mechanisms (Sakaba and Neher, 2001; Kaneko and Takahashi, 2004). According to Katsurabayashi et al. (2004), glycinergic eIPSCs are regulated via a PKA-dependent pathway, while sIPSCs are modulated directly by cAMP and/or PKA-independent pathways. Therefore, previous reports suggest that

DCOIT may differentially modulate intracellular release mechanisms in evoked vs. spontaneous synaptic transmission.

#### 4.2. Some technical aspects

Usually, action-potential evoked eIPSCs recorded at a single synapse level show large variance from 0 to about 200 pA in current amplitudes as shown in a case of Fig. 1A. Here, we have to remind that a single neuronal cell body receives many excitatory or inhibitory synaptic inputs from a few hundred to thousand. Therefore, eIPSC and eEPSC of previous reports recorded in brain slice preparation are the averaged current amplitude of many input currents. Consequently, these synaptic currents of slice preparation show almost steady current amplitude. Please note that there is large difference between the current amplitude variance between currents recorded from single synapses in our synapse-bouton preparation and from multi synapses in conventional slice preparation.

Since we calculated the average current amplitude of all currents including full failure, we can compare statistically the current difference between control and the presence of DCOIT.

The concentration response curves for either eEPSC or eIPSC are not unusual. Because, DCOIT facilitates transmitter release at low concentration but inhibits the release at high concentration. DCOIT at high concentration induces the depletion of transmitter by acting transmitter release machinery in nerve endings, as seen in Fig. 5A. Similar observations were reported in our previous report (Wakita et al., 2015) and Juang and Yonemura (1975) using MetHg.

#### 4.3. Toxicological implications

DCOIT is an alternative antifouling biocide that may have a significant impact on ecosystems (Guardiola et al., 2012). Toxic actions of DCOIT and other isothiazolone biocides have previously been reported (Williams, 2007; Thomas and Brooks, 2010). However, apparent neurotoxic actions of DCOIT have not been reported until now. We observed that DCOIT

significantly facilitates synaptic transmission mediated by GABA and glutamate in rat hippocampal CA3 neurons (Figs. 3–5). In addition, DCOIT may potentiate the transmission mediated by acetylcholine, as it inhibits acetylcholinesterase activity (Chen et al., 2014a). Thus, DCOIT is thought to affect both inhibitory and excitatory transmission. DCOIT is found in harbors, bays, and coastal areas worldwide (Steen et al., 2004; Harino et al., 2012; Lee et al., 2015; Mochida et al., 2015). The concentration of DCOIT in marine sediment varies from ‘not detected’ to several hundred  $\mu\text{g}/\text{kg}$ . Therefore, it may not be surprising to find local high concentrations of DCOIT everywhere. In the product safety assessment released from the Dow Chemical Company (2012), DCOIT is suggested to have a low risk of accumulating in the food chain. It is difficult to suggest the in vivo neurotoxicity based on our in vitro results, as the in vitro concentrations of DCOIT that had significant effects on synaptic transmission were 1–10  $\mu\text{M}$  (equivalent values; 282–2,822  $\mu\text{g}/\text{L}$ ). Thus, the changes in fundamental transmission may represent the potential for neurotoxic effects. In a study by Chen et al. (2014b), the marine medaka (*Oryzias melastigma*) was exposed to DCOIT at 9 nM (2.55  $\mu\text{g}/\text{L}$ ) for 28 days in order to examine proteomic changes in brain tissue. eEPSCs and eIPSCs were immediately affected by 0.03  $\mu\text{M}$  and 0.1  $\mu\text{M}$  (equivalent values; 8.4  $\mu\text{g}$  and 28.1  $\mu\text{g}$ ) DCOIT, respectively (Fig. 2). Long-term exposure to lower concentrations of DCOIT may change neurotransmission and result in neurotoxicity. The concentrations of DCOIT ranged from 0.04  $\mu\text{g}/\text{kg}$  to 281  $\mu\text{g}/\text{kg}$  dry sediment collected from Ostuki Bay, Japan (Hirano et al., 2007), Hiroshima Bay, Japan (Tsunemasa and Yamazaki, 2014; Mochida et al., 2015), and Busan Bay and Coast, South Korea (Kim et al., 2015a,b). The octanol-water partition coefficient of DCOIT is reported to be 4.5 (Shade et al., 1993) or 2.85 (Willingham and Jacobson, 1996). Thus, there is a possibility that DCOIT accumulates in organisms.

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**Conflict of interest statement**

We have no conflicts of interests to declare.

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### Figure Captions

**Figure 1.** Effects of 0.3 and 10  $\mu\text{M}$  DCOIT on GABAergic eIPSCs (A, B) and glutamatergic eEPSCs (C, D) evoked by paired-pulse focal electrical stimuli. Representative eIPSCs evaluated by paired-pulse stimulation before (1), during (2), and after (3) adding 0.3 (Aa) and 10  $\mu\text{M}$  DCOIT (Ba). Current traces 1, 2, and 3 were obtained at time points 1, 2, and 3 in (Ab) and (Bb), respectively. Each arrowhead indicates the peak of the  $P_1$  (filled arrowhead) and  $P_2$  (open arrowhead) eIPSC current amplitudes. Plots of the  $P_1$  current amplitudes of eIPSCs against time in the absence (open circle) and presence (gray circle) of 0.3 (Ab) and 10  $\mu\text{M}$  DCOIT (Bb). Electrical stimuli that failed to evoke eIPSCs (i.e., amplitude = 0) were counted as failures and used to calculate the failure rate (Rf) of eIPSCs. Typical eEPSCs before (1), during (2), and after (3) the application of 0.3 (Ca) and 10  $\mu\text{M}$  DCOIT (Da). Typical time courses of the  $P_1$  amplitude of eEPSCs before, during, and after the application of 0.3 (Cb) and 10  $\mu\text{M}$  DCOIT (Db).

**Figure 2.** Mean relative  $P_1$  current amplitude (A),  $P_1$  Rf (B), and PPR (C) of eIPSCs (gray bars) and eEPSCs (solid bars) evaluated by paired-pulse focal stimulation during the application of DCOIT at various concentrations (0.03–10  $\mu\text{M}$ ). Each bar represents mean  $\pm$  standard error of the mean (SEM). Data were obtained from 5–9 neurons. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

**Figure 3.** Effects of DCOIT on GABAergic sIPSCs and glutamatergic sEPSCs. Typical time courses of sIPSC (Aa) and sEPSC (Ba) frequencies before, during, and after adding 10  $\mu\text{M}$  DCOIT, indicated by the black bar. Typical current traces - 1 (control), 2 (10  $\mu\text{M}$  DCOIT), and 3 (washout) - of sIPSCs (Ab) and sEPSCs (Bb), obtained from the corresponding time points of 1, 2, and 3 in (Aa) and (Ba), respectively. (C) Mean relative frequencies (Freq., a), amplitudes (Amp., b), and decay times (c) of sIPSCs (gray bars) and sEPSCs (solid bars) during the application of DCOIT at various concentrations (0.03–10  $\mu\text{M}$ ). All parameters were normalized to values obtained in the absence of DCOIT. Each bar represents the mean  $\pm$  SEM of data obtained from 4 to 9 neurons. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

**Figure 4.** Effects of DCOIT on GABAergic mIPSCs and glutamatergic mEPSCs. Typical time courses of

mIPSC (Aa) and mEPSC (Ba) frequencies in control conditions before, during, and after the application of 10  $\mu\text{M}$  DCOIT (black bar) in the presence of 300 nM TTX in a  $\text{Ca}^{2+}$ -free external solution (gray bar). Typical current traces - 1 (control), 2 (TTX +  $\text{Ca}^{2+}$ -free), and 3 (TTX +  $\text{Ca}^{2+}$ -free + 10  $\mu\text{M}$  DCOIT) - of mIPSCs (Ab) and mEPSCs (Bb), obtained from the corresponding time points 1, 2, and 3 in (Aa) and (Ba), respectively. (C) Mean relative frequencies (Freq., a) and amplitudes (Amp., b) of mIPSCs (gray bars) and mEPSCs (solid bars) under control conditions and in the presence of 300 nM TTX +  $\text{Ca}^{2+}$ -free and 300 nM TTX +  $\text{Ca}^{2+}$ -free + 10  $\mu\text{M}$  DCOIT, respectively. Each bar represents the mean  $\pm$  SEM of data obtained from 5 neurons. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

**Figure 5.** Effects of repetitive (3 times) DCOIT application on glutamatergic mEPSCs. A typical time course of mEPSC frequency in control conditions and before, during, and after the repetitive (3 times) application of 10  $\mu\text{M}$  DCOIT (black bar) in the presence of 300 nM TTX in a  $\text{Ca}^{2+}$ -free external solution (gray bar). (B) Mean relative frequencies (Freq., a) and amplitudes (Amp., b) of mEPSCs under control conditions and in the presence of 300 nM TTX +  $\text{Ca}^{2+}$ -free and 300 nM TTX +  $\text{Ca}^{2+}$ -free + 10  $\mu\text{M}$  DCOIT 1, DCOIT 2, and DCOIT 3, respectively. Each bar represents the mean  $\pm$  SEM of data obtained from 5 neurons. \*  $P < 0.05$ , \*\*\*  $P < 0.001$ .

**Figure 6.** Effects of DCOIT on  $I_{\text{Na}}$ .  $I_{\text{Na}}$  was elicited by a test pulse from a  $V_{\text{H}}$  of  $-70$  mV to  $-20$  mV. Typical current traces of  $I_{\text{Na}}$  during and after the application of 3 and 10  $\mu\text{M}$  DCOIT (A). The effects of various concentrations of DCOIT on the peak amplitudes (Amp) of the inward currents are expressed relative to the amplitude of the current response obtained in the absence of DCOIT (Ba). The effects of various concentrations of DCOIT on the decay times (90% to 37%) of the inward currents are expressed relative to the decay time of the current response obtained in the absence of DCOIT (Bb). Each bar represents the mean  $\pm$  SEM of data obtained from 4 to 7 neurons.

Figure 1

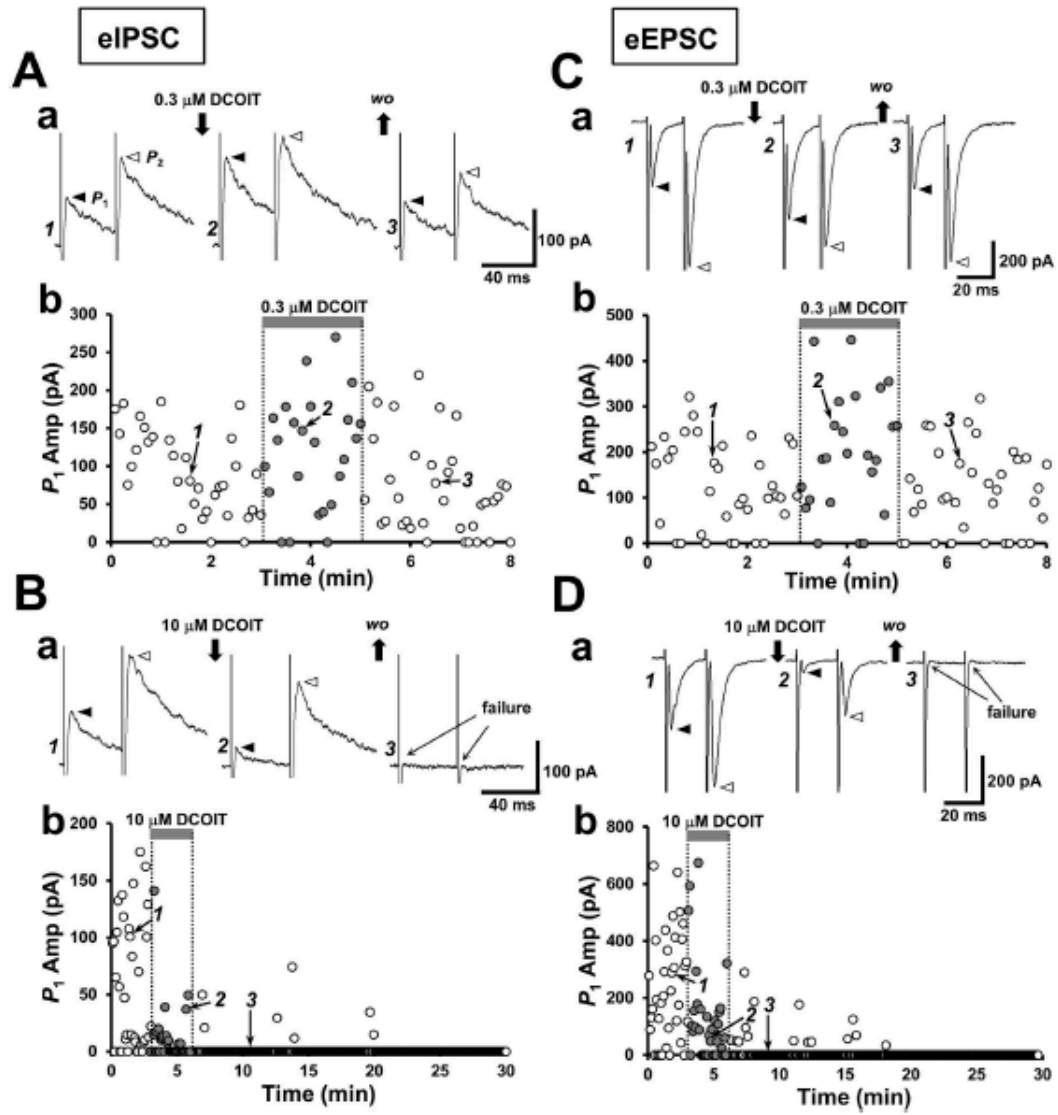


Figure 2

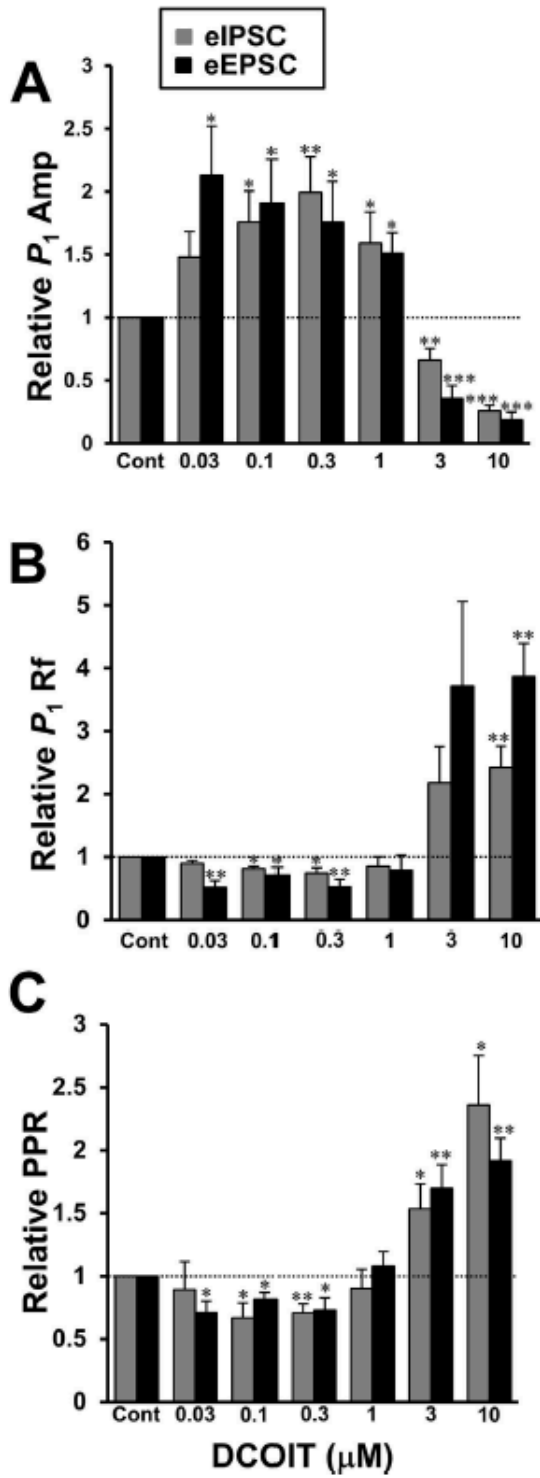


Figure 3

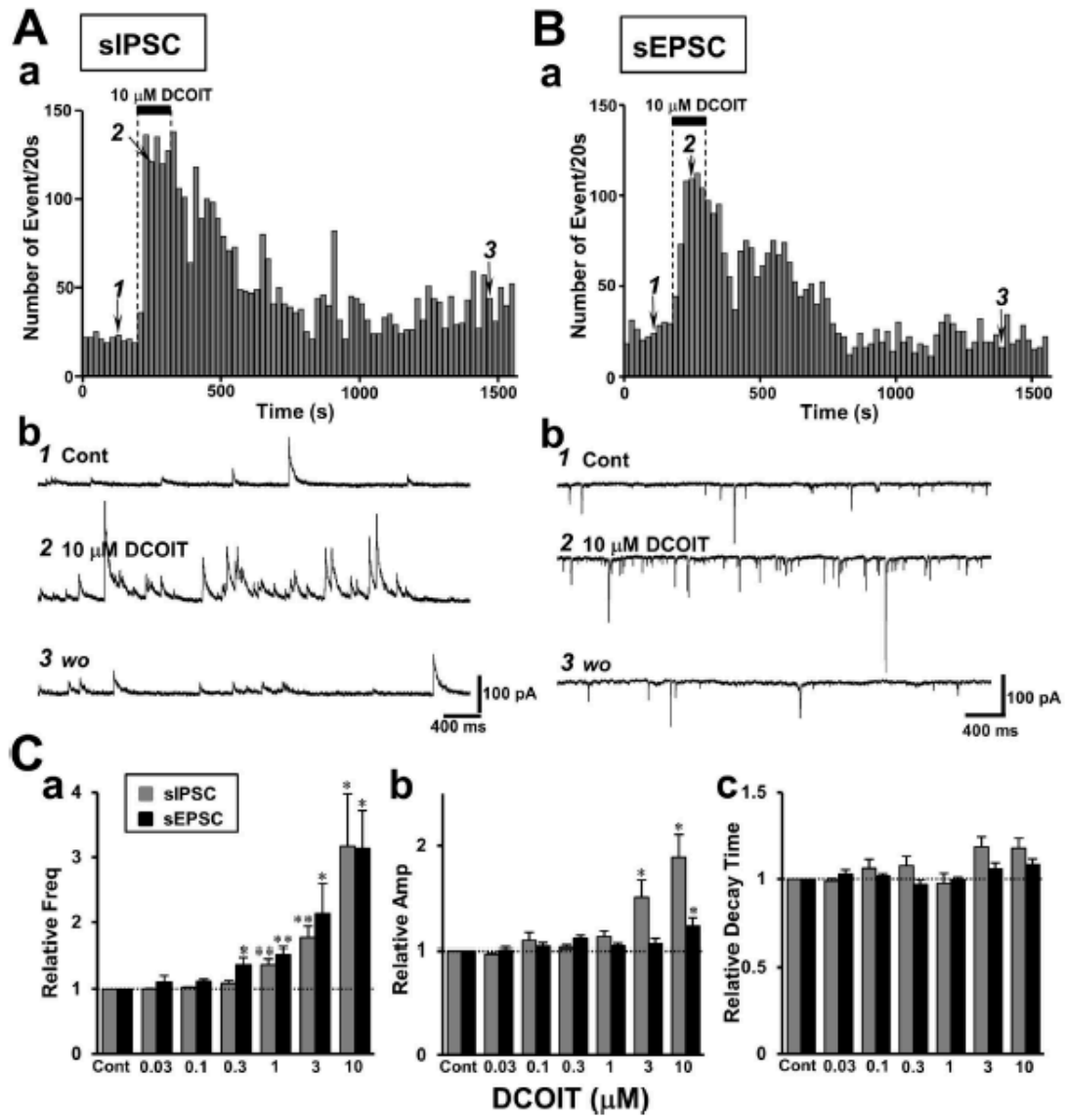




Figure 4

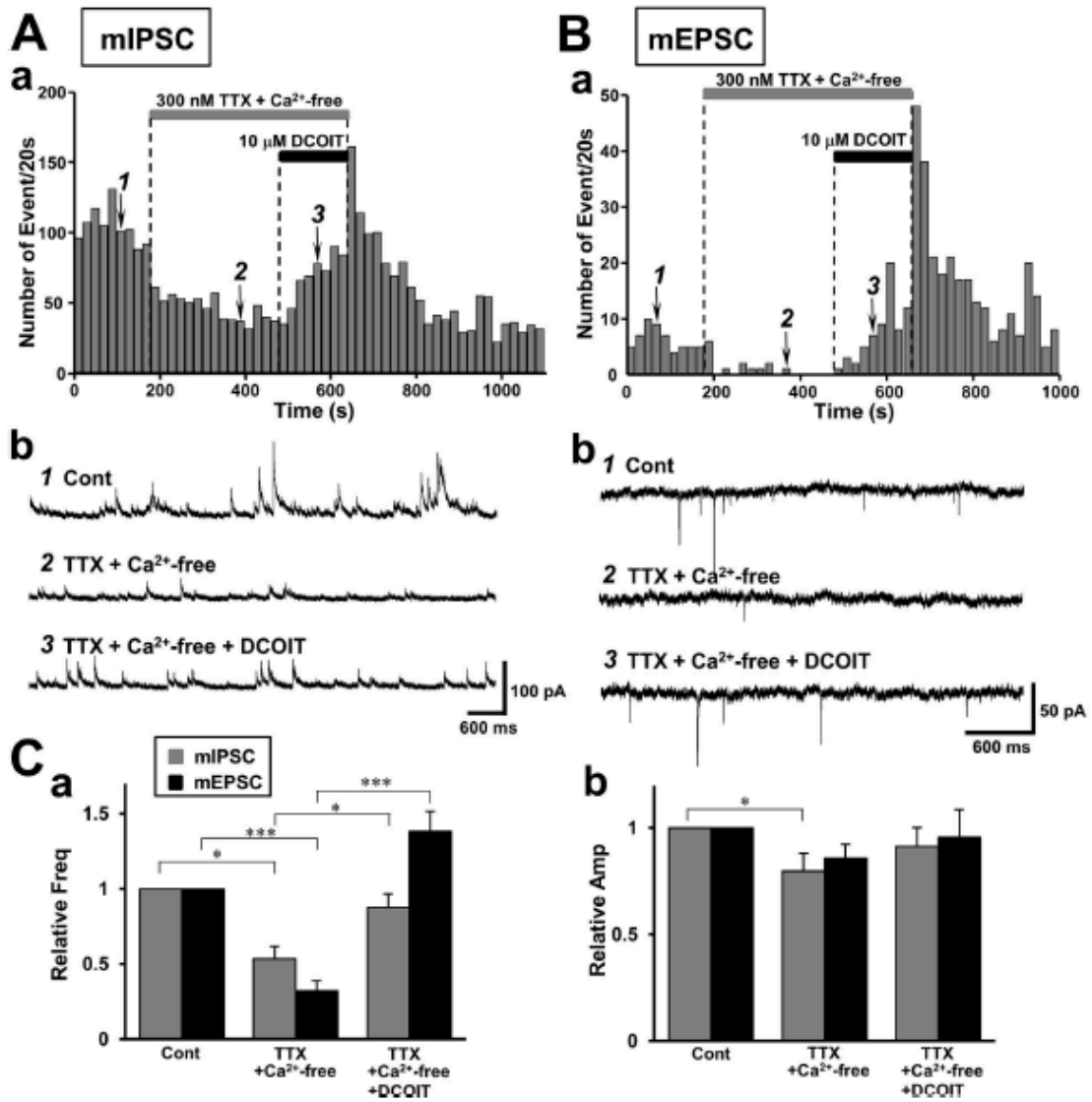


Figure 5

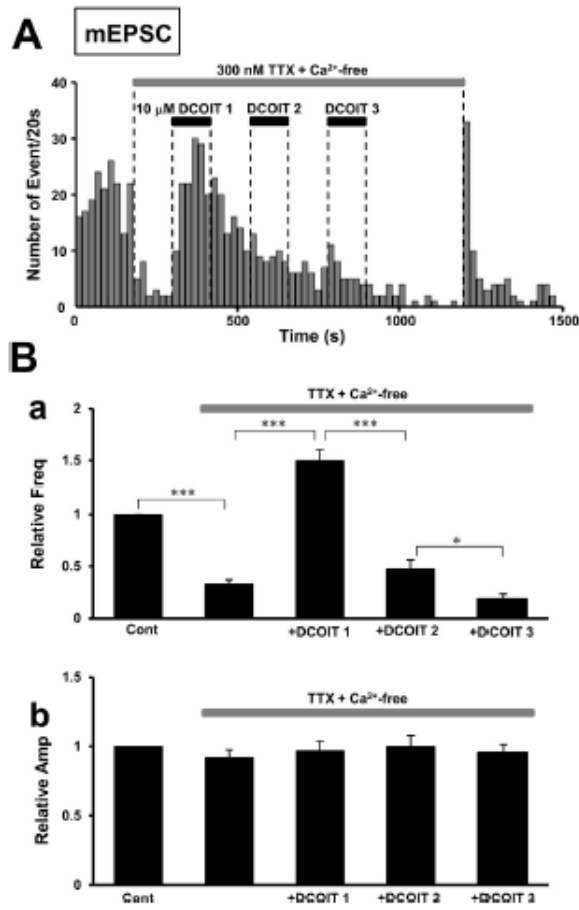


Figure 6

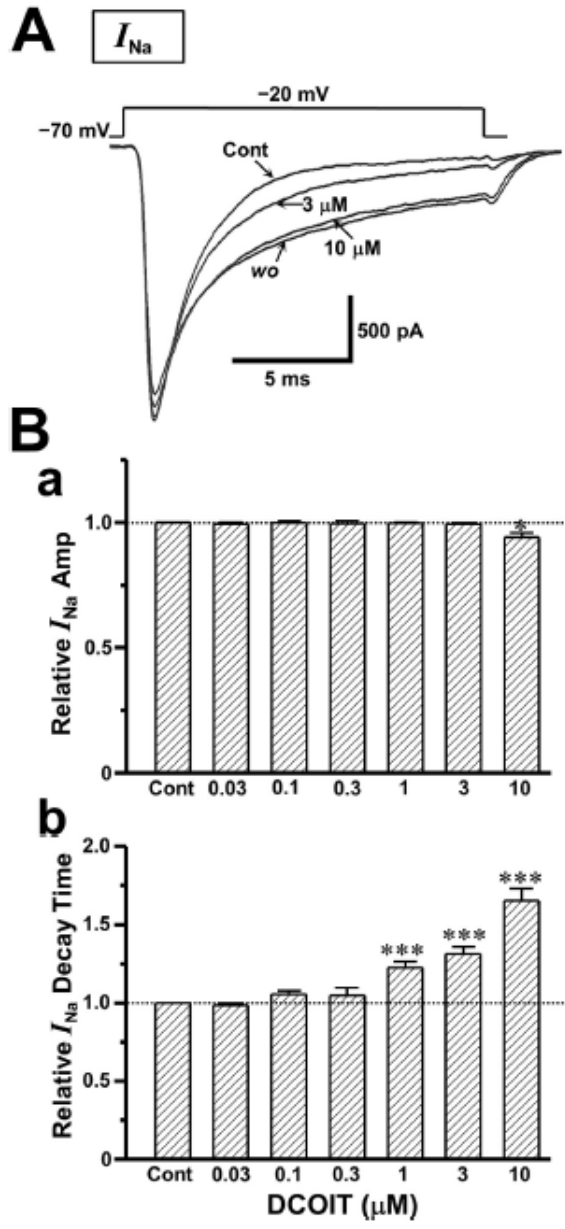


Table 1

Solutions for recording currents elicited by GABA and glutamate.

Recording Currents	External Solution	Internal Pipette Solution
<b>GABAergic IPSC and <math>I_{GABA}</math></b>		
Ionic Composition	150 mM NaCl 5 mM KCl 2 mM $CaCl_2$ 1 mM $MgCl_2$ 10 mM Glucose 10 mM HEPES pH 7.4 Adjusted with Tris base	5 mM CsCl 135 mM Cs-methanesulfonate 5 mM TEA-Cl 10 mM EGTA 10 mM HEPES 4 mM ATP-Mg pH 7.2 Adjusted with Tris base
<b>Glutamatergic EPSC and <math>I_{Glu}</math></b>		
Ionic Composition	150 mM NaCl 5 mM KCl 2 mM $CaCl_2$ 1 mM $MgCl_2$ 10 mM Glucose 10 mM HEPES pH 7.4 Adjusted with Tris base	5 mM CsCl 135 mM CsF 5 mM TEA-Cl 2 mM EGTA 10 mM HEPES 5 mM QX-314 bromide pH 7.2 Adjusted with Tris base

Table 2

Solutions for recording voltage-dependent currents.

Recording Current	External Solution	Internal Pipette Solution
<b>Voltage-dependent Na<sup>+</sup> Current (<math>I_{Na}</math>)</b>		
Ionic Composition	60 mM NaCl 100 mM Choline -Cl 10 mM CsCl 10 mM glucose 0.01 mM LaCl <sub>3</sub> 5 mM TEA-Cl 10 mM HEPES pH 7.4 Adjusted with Tris base	105 mM CsF 30 mM NaF 5 mM CsCl 5 mM TEA-Cl 2 mM EGTA 10 mM HEPES 2 mM ATP-Mg pH 7.2 Adjusted with Tris base
<b>Ba<sup>2+</sup> Current (<math>I_{Ba}</math>) through Voltage-dependent Ca<sup>2+</sup> Channels</b>		
Ionic Composition	145 mM Choline-Cl 5 mM CsCl 5 mM BaCl <sub>2</sub> 1 mM MgCl <sub>2</sub> 10 mM glucose 10 mM HEPES pH 7.4 Adjusted with Tris base	80 mM Cs-methanesulfonate 60 mM CsCl 5 mM TEA-Cl 2 mM EGTA 10 mM HEPES 2 mM ATP-Mg pH 7.2 Adjusted with Tris base