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Effects of a red-tide toxin on fish hearing

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Abstract Red tides are formed from blooms of marine algae. Among them, the dinoflagellate (*Karenia brevis*) that is responsible for Florida red tides can release many types of natural toxins, which cause massive kills of marine animals, including endangered species, and threaten human health. This study was to investigate whether or not a neurotoxin, brevetoxin-3, purified from Florida red tides affects hearing sensitivity of a teleost fish, the goldfish (*Carassius auratus*). LD₅₀ of the goldfish that were intraperitoneally injected with brevetoxin-3 was 0.068 µg g⁻¹. Evoked auditory brainstem responses were recorded, and hearing threshold was determined using a correlation method. By comparing thresholds of fish before and after a sublethal-dose injection (0.064 µg g⁻¹) of the toxin, we found that brevetoxin-3 significantly reduces auditory sensitivity up to 9 dB at low frequencies (100 Hz and 500 Hz), but not at a high frequency (2,000 Hz). Reduction of hearing sensitivity was recovered within 24 h. To our knowledge, this is the first study showing a natural red-tide toxin causes minor hearing loss in vertebrates. Results of the study indicate that brevetoxin-3 could affect hearing capabilities of marine animals that survived exposure to red tides. Mechanisms of the toxin-induced reduction of hearing sensitivity are discussed.

Keywords Audiogram · Auditory brainstem response · Brevetoxin · Goldfish · Neurotoxin

Introduction

Red tides – patches of discolored seawater – have been reported worldwide (Okaichi et al. 1987). They are formed by blooms of marine phytoplankton populations, most of which are dinoflagellates and diatoms. Among these marine algae, the dinoflagellate, *Karenia brevis* Davis (formerly *Gymnodinium breve* or *Ptychodiscus brevis*), has been identified as the cause of the red tides occurring in the Gulf of Mexico along the coastline from Texas to Florida (Davis 1948; Wilson and Ray 1956; Steidinger 1979). The Gulf Stream can carry *K. brevis* to the east coast of Florida and up to the North Carolina coast (Tester et al. 1988). *K. brevis* produces hemolytic and neurotoxic toxins (see Baden et al. 1995 for a review), which result in massive kills of fishes, birds, and marine mammals, including endangered manatees (Bossart et al. 1998). Red-tide toxins can also cause neurotoxic shellfish poisoning (NSP) in humans who consume shellfish contaminated with *K. brevis* toxins (Baden et al. 1995). Although NSP is not life threatening, humans intoxicated by red-tide toxins show various symptoms, including dizziness, paraesthesia, nausea, diarrhea, and vomiting. In addition, the inhalation of wind-sprayed red-tide toxins leads to respiratory irritation, copious rhinorrhea, and non-productive cough. In general, red tides have dramatic impacts on the environment, local economy, and human health.

Neurotoxins released by *K. brevis* are known as brevetoxins, which are thought to be responsible for massive fish kills and human NSP. Brevetoxin-3 (formerly GB-3 by Chou and Shimizu 1982; T17 by Baden et al. 1979; or PbTx-3 by Poli et al. 1986) is one of ten types of neurotoxins that have been purified from laboratory cultures of *K. brevis* (Baden and Mende 1982; Baden 1989; Baden et al. 1995). Brevetoxin-3 is an activator of voltage-gated Na⁺ channels. It opens the

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sodium channels in the neuron membrane at the resting potential, resulting in an excessive influx of Na^+ that leads to a blockage of neuronal excitation (Baden 1983; Huang et al. 1984; Poli et al. 1986). This mechanism may account for respiratory failure that leads to fish kills, and respiratory irritation and NSP in humans.

Although previous studies have demonstrated brevetoxic effects on the respiratory system, heart, and neuromuscular junction (Sasner et al. 1972; Baden et al. 1982, 1984; Bossart et al. 1998), no study has been reported regarding possible effects of any red-tide toxin on sensory systems. Fish intoxicated by red tide toxins show corkscrew swimming and loss of balance, indicating that their auditory/vestibular systems could be affected by the neurotoxins. The present study was to investigate whether or not brevetoxin-3 affects hearing sensitivity of a teleost fish. Preliminary data of this study were reported in abstract form (Lu and Xu 2001).

Methods and materials

Animal preparation

Twenty-five goldfish, *Carassius auratus* (total length 100–120 mm) were used in this study. Fish were obtained from a local fish distributor and housed in several 15-gallon tanks containing water that was aerated, filtered, treated with Ammo Lock 2 or AmQuel (That Fish Place, Lancaster, Pa., USA), and kept at approximate 20 °C. Each fish was anesthetized in a bath of 0.05% MS-222 solution and then immobilized by an intramuscular injection of Flaxedil ($1 \mu\text{g g}^{-1}$ body weight) in the tail. The anesthetized fish was placed in a holder and then submerged in water in an experimental tank except for the top of its head. A small tube was inserted into the fish's mouth, and its gills were irrigated with air-saturated water at a flow rate of 100 ml min^{-1} throughout experiments. After a small piece of skin ($3 \text{ mm} \times 3 \text{ mm}$) was surgically removed to expose the skull, a piece of paper towel soaked in 0.7% NaCl was placed on the cranium. The fish remained undisturbed in the holder for at least 1 h before neural recording collection in order to let it fully recover from anesthesia.

LD₅₀ experiments

Brevetoxin-3 was purchased from Calbiochem-Novabiochem (San Diego, Calif., USA). It was pre-dissolved in 20 μl methanol and then diluted with 0.7% phosphate-buffered NaCl. In order to obtain the lethal dose, LD₅₀ experiments were carried out on the goldfish with an intraperitoneal injection of the neurotoxin using a 50- μl syringe. We determined the LD₅₀ using the up-and-down method proposed by Dixon (1965). Briefly, a fish was injected with a dose of the toxin. If the fish died within 24 h, next fish was injected with a lower dose. If that fish survived for 24 h, the next fish would be injected with a higher dose. This procedure was repeated on different fish until a sample size of six was reached. The original dose was $0.07 \mu\text{g g}^{-1}$ body weight, and the dose increment/decrement was set constant ($0.0064 \mu\text{g g}^{-1}$). Since this method uses a small sample size, it requires fewer fish than traditional LD₅₀ methodology.

Neurophysiological setup

The experimental setup, modified from Lu and Fay (1993), comprised an experimental tank, computer, power amplifier, preamplifiers, and modular system made by Tucker-Davis Technologies

(TDT). A UW-30 underwater loudspeaker was electrically shielded, placed on the bottom of the tank, and embedded in sand. The distance between the loudspeaker and the fish was about 150 mm. Seven tones were 100, 200, 500, 1000, 2000, 3350, and 5000 Hz. Durations of the acoustic signals were 20.0, 10.0, 10.0, 5.0, 2.5, 2.4, and 1.6 ms; rise and fall times were 0, 0, 4.0, 2.0, 1.0, 0.6, and 0.4 ms. For toxin experiments, 100-, 500-, and 2000-Hz signals were used. These signals were synthesized by the computer, read out of a digital-to-analog converter at 20 kHz, attenuated by a programmable attenuator (PA4), and sent to a Hafler power amplifier. The output of the power amplifier was fed to the UW-30 loudspeaker. TDT SigGen, SigCal, and BioSig software was used for signal synthesis, experimental tank calibration, and data acquisition. Sound pressure levels were calibrated using a Reson hydrophone (TC 4013, sensitivity = $-211 \pm 3 \text{ dB re } 1 \text{ V}/\mu\text{Pa}$) suspended in the tank in the position normally occupied by the fish. All sound levels were reported in decibels with respect to $1 \mu\text{Pa RMS}$.

Auditory brainstem recording

Evoked auditory responses from the brainstem were recorded using a pair of 0.125-mm silver wires that were Teflon coated except at the tip regions (Corwin et al. 1982; Kenyon et al. 1998; Lu and Xu 2002). The recording electrode was placed on the wet paper towel on the fish's skull; the reference electrode was kept in the trunk muscle. Auditory brainstem recording (ABR) responses with 45-ms durations were filtered between 10 Hz and 10,000 Hz and amplified 100,000 times by two Grass AC preamplifiers (P55), and digitally recorded at a sampling rate of 20 kHz. Since the amplitude of evoked auditory responses was small, recorded neural activities were averaged 2,000 times to extract ABR responses by reducing the background noise. In order to eliminate potential stimulus artifacts, acoustic signal starting phases were alternated by 180°.

Threshold determination

Since the amplitude of ABR waveforms reduces as the stimulus intensity decreases, it is difficult to determine auditory threshold without biases based upon eyeball monitoring of ABR waveforms. We used a statistical method of correlation to distinguish neural response from background noise. Two sweeps of ABR responses, each of which was averaged 2,000 times, were collected in response to the same acoustic stimulus. The correlation coefficient (R), ranging from 0 to 1, was calculated by comparing the two ABR sweeps. The recording durations used for the correlation analysis were twice the stimulus durations (40 ms at 100 Hz, 20 ms at 500, and 10 ms at 2,000 Hz). The criterion of $R = 0.3$ was selected to determine hearing threshold (see Results).

Results

Goldfish audiograms have been obtained in several laboratories using different methods (see Fay 1988 for a review). Figure 1 shows a comparison of goldfish audiograms using respiratory classical conditioning and ABR methods. There is a close match of our audiogram and the behavioral audiogram (Fay 1969) above 1 kHz. However, our ABR audiogram is less sensitive than the behavioral audiogram below 1 kHz. This could be because low-frequency sounds are less effective at generating evoked ABR responses than high-frequency sounds. In addition, our ABR thresholds are about 10 dB higher across the frequencies than those reported by Kenyon et al. (1998). These threshold differences may derive from different criteria used for threshold determination in the studies.

Figure 2 illustrates ABR waveforms recorded from a goldfish, showing that ABR responses increase monotonically with stimulus intensity at different frequencies. At low frequencies (i.e., 100 Hz and 500 Hz), ABR response frequency was about twice the stimulus frequency. However, at a high frequency (i.e., 2,000 Hz), the fast-Fourier-transform peak of ABR responses tended to be lower than double the stimulus frequency (not shown in Fig. 2).

The correlation coefficient (R) was used to determine auditory threshold. Figure 3 shows that R of ABR responses increases with stimulus intensity. The response curves tend to scatter and have small slopes for $R < 0.3$,

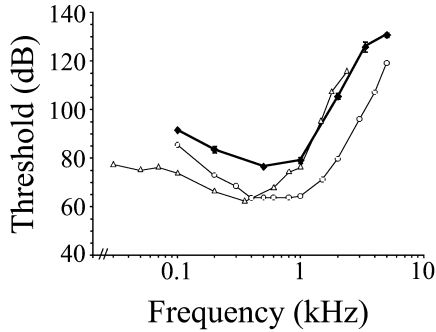


Fig. 1 Comparison of goldfish audiograms obtained using different methods. *Diamonds*: the present study ($n = 9$, vertical bars are standard errors); *triangles*: data from Fay (1969); and *circles*: data from Kenyon et al. (1998)

Fig. 2 Examples of auditory brainstem recording (ABR) waveforms recorded at three frequencies from a goldfish. The stimulus waveforms at the bottom were recorded from the hydrophone and averaged 2,000 times

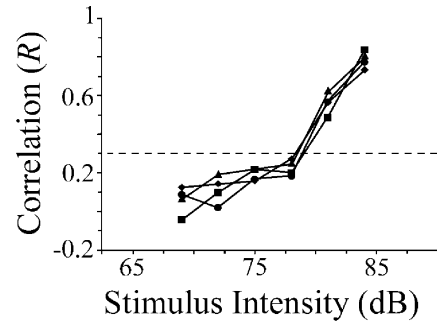
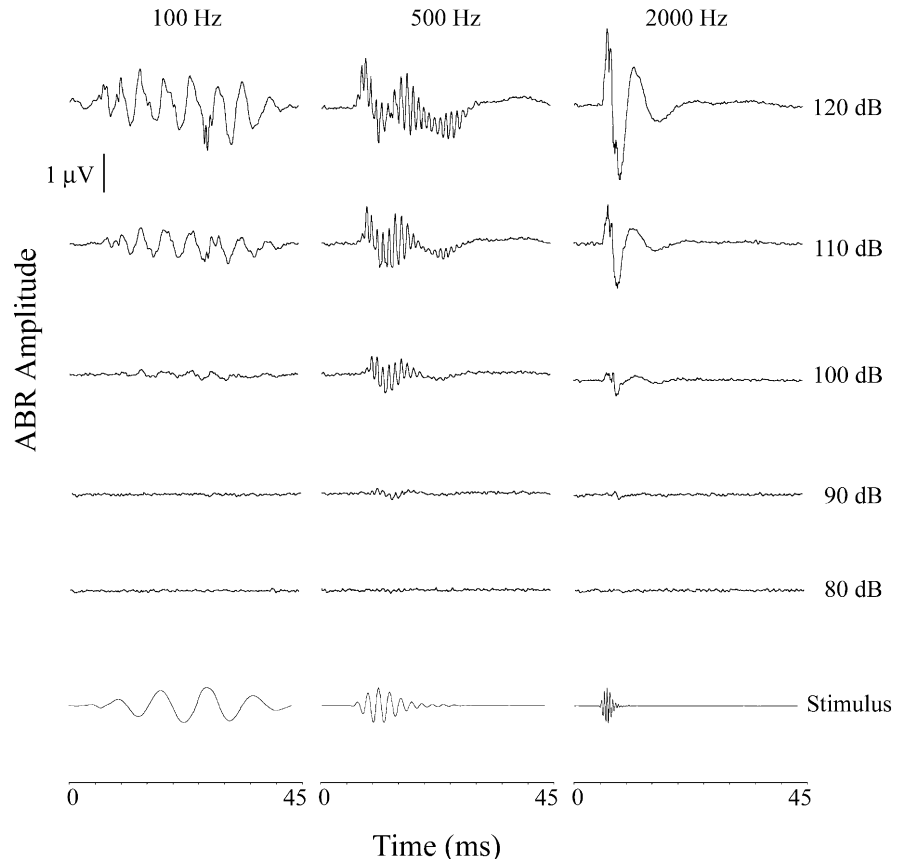


Fig. 3 Correlation versus stimulus intensity functions at 500 Hz for four repeated measurements. The *dashed line* indicates the criterion correlation coefficient $R = 0.3$ for threshold determination

while the curves overlap closely and have steep slopes for $R > 0.3$. Based on these response characteristics, the criterion of $R = 0.3$ was selected for threshold determination.

Control experiments were conducted to rule out potential effects of other factors rather than the toxin on hearing sensitivity (Fig. 4). There was no significant difference in threshold for goldfish before and after receiving intraperitoneal injections of 0.7% NaCl (upper panel; two-tailed Student's t -test, $P > 0.05$). In addition, no threshold difference was found for goldfish before and after having injections of a methanol solution (lower panel; two-tailed Student's t -test, $P > 0.05$). This solu-

tion had the same volume but a methanol concentration 1,000 times higher than that in the toxin solution.

K. brevis is about 20–40 μm long and 10–15 μm thick and moves in the water at 0.064 mm s^{-1} using its two flagella. It has chloroplasts for photosynthesis. *K. brevis* is 1 of approximately 40 marine algae found in the Gulf of Mexico that can release natural toxins. Brevetoxin-3 released by *K. brevis* is a lipophilic 11-ring polyether with a molecular weight of 897.1 (Fig. 5A). From the experimental data obtained using the up-and-down method (Fig. 5B), the 24-h LD_{50} of the toxin for the goldfish was calculated to be 0.068 $\mu\text{g g}^{-1}$.

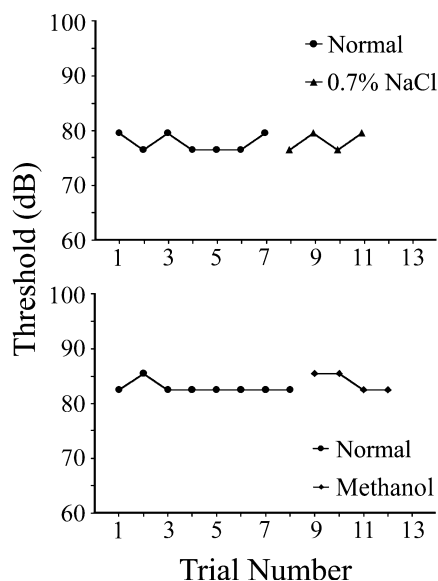


Fig. 4 *Upper*: threshold versus trial number for a goldfish before and after an injection of 0.7% NaCl solution; *lower*: threshold versus trial number for another goldfish before and after a methanol injection. The inter-trial interval was approximately 30 min. Stimulus frequency 500 Hz

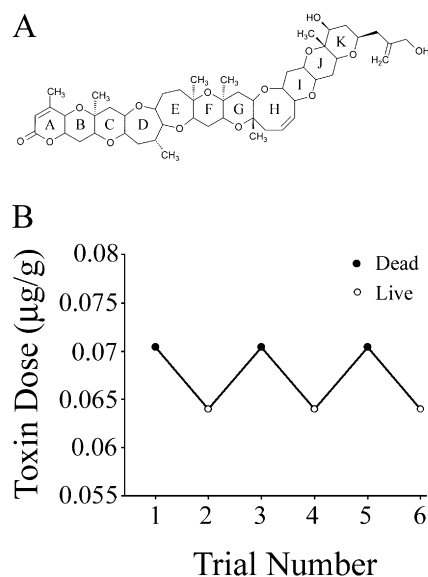


Fig. 5 **A** Chemical structure of brevetoxin-3. **B** Brevetoxin-3 dose versus trial number. Stimulus frequency 500 Hz

Brevetoxin-3 reduced the amplitude of the ABR response, but did not alter the feature of doubling of stimulus frequency at 100 Hz and 500 Hz (Fig. 6). We found that toxic effects on ABR response amplitude are stimulus intensity dependent. At suprathreshold (e.g., 84 dB in Fig. 6), the toxin resulted in a decrease of ABR responses by 19%. Toxin-induced reduction of ABR responses gradually increases as stimulus level decreases down to 78 dB re 1 μPa . However, the reduction rate appears to decrease as stimulus intensity continues to fall to and below the normal threshold, 73.5 dB re 1 μPa .

Figure 7 shows an example of the toxin-induced reduction of hearing sensitivity of a goldfish. The fish was injected with a toxin dose (i.e., 0.064 $\mu\text{g g}^{-1}$), which was 6% below the LD_{50} . The threshold started to increase within 30 min after the toxin injection, and a 9-dB hearing loss was observed. The threshold up-shift often held up to

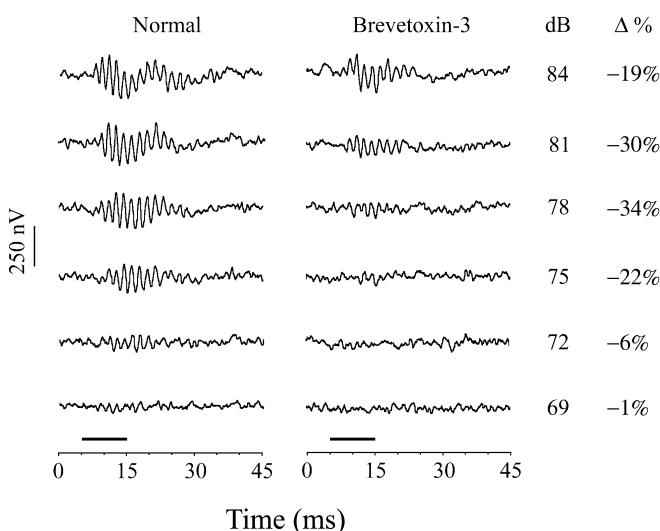


Fig. 6 Effects of the toxin on the amplitude of ABR waveforms at 500 Hz. $\Delta\% = (V_{\text{toxin}} - V_{\text{norm}}) / V_{\text{norm}}$, where V_{norm} and V_{toxin} are the amplitudes of ABR recordings before and after a toxin injection, calculated in nanovolts RMS. Horizontal bars above time axes indicate the stimulus duration of 10 ms. $\text{Threshold}_{\text{norm}} = 73.5 \text{ dB re } 1 \mu\text{Pa}$, and $\text{threshold}_{\text{toxin}} = 82.5 \text{ dB re } 1 \mu\text{Pa}$

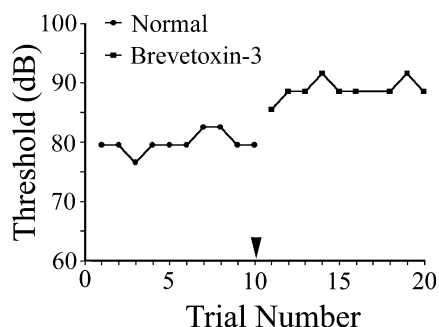


Fig. 7 Effects of the toxin on hearing sensitivity of a goldfish in response to 500-Hz stimuli. The inter-trial interval was approximately 30 min. The arrowhead indicates the time of toxin injection, i.e., immediately after the recording of the tenth trial

18 h and then returned to the normal threshold level. Figure 8 shows threshold reduction and recovery of a goldfish. Swimming behavior of all fish used for hearing measurements was completely recovered within 24 h.

Effects of brevetoxin-3 on hearing sensitivity were tested at 100, 500, and 2,000 Hz for five goldfish. At these three frequencies, average normal thresholds (\pm SE) were 93.3 ± 1.8 , 75.3 ± 1.5 , and 97.1 ± 2.7 dB, and thresholds with the toxin injection were 96.3 ± 2.0 , 81.2 ± 1.6 , and 98.0 ± 2.3 dB (see Fig. 9). There is significant threshold difference between normal and experimental fish (two-factor ANOVA, $P < 0.001$). The toxin significantly reduced hearing sensitivity at 100 Hz (Neuman-Keuls test, $P < 0.01$) and 500 Hz ($P < 0.001$), but not at 2,000 Hz ($P > 0.05$).

Discussion

LD₅₀ and toxic effects on behavior

Baden and Mende (1982) conducted 24-h LD₅₀ experiments on mice with different routes of administration of brevetoxin-3 (formerly T17). They found that intravenous administration was most lethal while oral administration was least toxic. The LD₅₀ of the goldfish is half the intraperitoneal LD₅₀ of mice ($0.17 \mu\text{g g}^{-1}$), indicating that fish are less tolerant to the toxin than mammals.

In this study we videotaped behavior of the goldfish in three conditions: normal, sublethal toxin injection ($< 0.064 \mu\text{g g}^{-1}$), and lethal injection ($> 0.07 \mu\text{g g}^{-1}$). The extent of abnormal swimming behavior of goldfish with a toxin injection was dose dependent. The goldfish receiving a lethal injection of the toxin reacted in similar ways as previously reported (Baden 1983), including crazy twisting, circular swimming, loss of balance and opercular movement, and complete paralysis. They all died within a day. It took longer time for the goldfish with a sublethal injection to show swimming abnormality, and the extent of abnormality was less serious than those with a lethal injection. All fish with a sublethal injection behaviorally recovered within 1–24 h, depending upon the dose. This correlates with the time-course of functional recovery of minor hearing loss.

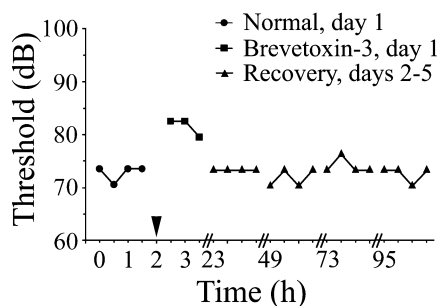


Fig. 8 Time-course of toxic effects on a goldfish in response to 500-Hz stimuli. Time started from the beginning of the first control recording with approximate inter-trial intervals of 30 min within a day. The *arrowhead* indicates the time of a toxin injection

The dose we used on fish for ABR recordings was equal to, or less than, $0.064 \mu\text{g g}^{-1}$, which was 6% less than the LD₅₀. All hearing threshold data presented in this paper were obtained from fish injected with a sublethal dose of brevetoxin-3, and they all survived and showed normal behavior after the experiments. Therefore, we rule out the possibility that the hearing reduction observed in this study resulted from toxin-induced health problems rather than from auditory causes. In other words, we did not record neural responses from dying or dead fish. Although the toxin indeed affects other organs/systems of fish, the hearing reduction does not appear to derive from secondary effects due to failure of other systems that were affected by the toxin.

Mechanisms of brevetoxin-induced hearing loss

Results of this study show that brevetoxin-3 affects the fish auditory system. However, the mechanisms of brevetoxin-induced hearing reduction are not known. Brevetoxin-3 caused dose-dependent depolarization of crayfish giant axons and rat brain cells, and this depolarization appeared to result from toxic effects on influx of Na^+ (Huang et al. 1984; Purkerson et al. 1999). Brevetoxin-3 depolarizes neurons at their resting potentials by opening voltage-gated Na^+ ion channels and also depresses fast inactivation of Na^+ channels, resulting in sustained depolarization. These effects lead to complete loss of excitation of neurons.

Previous studies have shown that brevetoxin-3 can bind to site 5 of the α -subunit of TTX-sensitive, voltage-gated Na^+ channels (Baden 1989; Jeglitsch et al. 1998). It appears that the K-ring side-chain of the toxin shown in Fig. 5A plays an important role in toxic effects of brevetoxin-3 on voltage-gated Na^+ channels (Purkerson-Parker et al. 2000). Minor modifications of the side chain did not change the binding and toxic effects. However, major structural changes of the side-chain diminished the toxic effects though they did not alter the binding to Na^+ channels. These derivatives were functionally antagonistic to brevetoxin-3.

In the present study, the toxin was intraperitoneally injected into fish. It is likely that the toxin was taken into the blood stream and carried to the auditory system

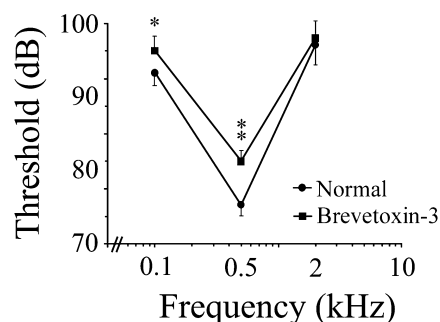


Fig. 9 Average auditory thresholds at three frequencies for control and experimental fish ($n = 5$). * $P < 0.01$, ** $P < 0.001$

including the periphery (ears and auditory nerves) and CNS. Since brevetoxin-3 has toxic effects on voltage-gated Na^+ channels, it could block conduction of neural signals along auditory nerves such as saccular, lagenar, and utricular nerve bundles in fishes. To our knowledge, no study has been reported regarding the effects of brevetoxins on sensory hair cells in animal species. Mechanically-gated K^+ channels in ciliary bundles and voltage-gated Ca^{2+} at the base of hair cells are critical for excitation and neurotransmitter release of hair cells. However, no experiment has been done to determine if the toxin affects these ion channels related to auditory transduction. Benson and Tischler (1999) reported brevetoxin-3 in the blood and brain tissue after rats inhaled the toxin, and their results indicated that the toxin could pass through the blood-brain barrier. In addition, several in vitro studies have demonstrated that brevetoxin-3 affects voltage-gated Na^+ channels in brain neurons of rats (Poli et al. 1986; Jeglitsch et al. 1998; Purkerson et al. 1999). Results of these studies lead to the hypothesis that brevetoxin-3 can affect the central auditory system of fishes as well as the auditory periphery.

Conclusions and future experiments

Results of this study show that a natural neurotoxin found in Florida red tides reduces hearing sensitivity of a teleost fish, and that the hearing reduction is reversible. This is the first study that demonstrates toxic effects of a red-tide toxin on a vertebrate sensory system. These findings indicate that marine animals that survive exposure to Florida red tides could suffer minor hearing loss.

Future experiments will focus on marine fish species that often encounter Florida red tides. Field and in vitro studies will help elucidate the effects of natural red tide toxins on auditory systems of vertebrates. Field studies will be carried out to determine whether red tide toxins cause hearing loss in fish in the natural environment. We will explore ABR recordings from fishes during red tide blooms. It would be also interesting to find out whether or not brevetoxins target sensory hair cells in fish otolithic organs, auditory nerves, and/or auditory neurons in the brain.

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