Neuroprotective Effects of Tetrodotoxin as a Na\(^+\) Channel Modulator and Glutamate Release Inhibitor in Cultured Rat Cerebellar Neurons and in Gerbil Global Brain Ischemia

Paul G. Lysko, PhD; Christine L. Webb, MS; Tian-Li Yue, PhD; Juan-Li Gu, MD; Giora Feuerstein, MD

Background and Purpose Studies examining the role of tetrodotoxin-sensitive ion channels in hypoxic-ischemic neuronal damage have concluded that sodium influx is an important initiating event. We examined the neuroprotectant effect of tetrodotoxin on both cultured cerebellar neurons and on CA1 hippocampal neurons of gerbils exposed to brain ischemia.

Methods We studied neuroprotective mechanisms using cultured rat cerebellar granule cells exposed to veratridine, which induced cytotoxicity, neurotransmitter release, and calcium influx. Survival of gerbil CA1 neurons was examined by direct neuron counts 7 days after 6 minutes of global ischemia with reperfusion.

Results Tetrodotoxin protected cultured neurons in a dose-dependent manner from veratridine-induced toxicity (protective concentration [PC\(_{50}\)]=22 nmol/L). Veratridine induced [H\(^+\)]aspartate efflux that was sodium dependent, only 25% calcium dependent, and was inhibited by tetrodotoxin (inhibitory concentration [IC\(_{50}\)]=60 nmol/L). Veratridine initiated increases in intracellular calcium that were also reversed by tetrodotoxin (IC\(_{50}\)=63 nmol/L); reversal was dependent on the sodium-calcium exchanger and the sodium-potassium pump. Neuroprotection of 90% (n=10; P=.001 versus vehicle) of gerbil CA1 hippocampal neurons was achieved by pretreatment with 2 ng of tetrodotoxin delivered three times intracerebroventriculally, without causing hypothermia.

Conclusions Sodium channel blockers like tetrodotoxin may have utility in treatment of ischemic neuronal injury by preventing excessive neuronal depolarizations, limiting excitotoxic glutamate release through reversal of the sodium-dependent glutamate transporter, preventing intracellular calcium overload, preserving cellular energy stores, and allowing recovery of ionic homeostasis through operation of the sodium-calcium exchanger. (Stroke. 1994;25:2476-2482.)

Key Words • cerebral ischemia • gerbils • glutamates • neuroprotection • rats
cell death induced by veratridine is not dependent on the cellular energy state and occurs in buffer containing glucose. Neurons (approximately $3 \times 10^4$) in 35-mm dishes were incubated at 37°C in the aforementioned buffer for 40 minutes before the addition of veratridine. The process consisted of a wash, 10-minute preincubation, and a 30-minute incubation, each in 1 mL of fresh buffer. Tetrodotoxin (Sigma Chemical Co) was added in increasing concentrations to separate dishes 20 minutes before the addition of veratridine (Research Biochemicals International). Sixty minutes after the addition of veratridine, cells were assessed for viability by staining with fluorescein diacetate (Sigma), which had been freshly diluted 1/1000 into incubation buffer from a stock of 10 mg/mL aceto. The staining solution (1 mL) was aspirated after 5 minutes and replaced with fresh buffer for immediate counting of viable neurons by fluorescence microscopy. Viability is expressed as a percentage of the total number of cells retaining fluorescein. Viability data are averaged duplicate determinations from six separate titrations, using three different neuronal preparations prepared on separate days.

For neurotransmitter release experiments, neurons in 35-mm dishes were washed in buffer as above and incubated in 1 mL buffer with 1 μCi of $[^3H]$Hapartic acid (NET-581, DuPont NEN Research Products) for 15 minutes at 37°C, essentially as previously described by Gallo et al.15 Labeled neurons were rapidly washed twice in buffer and incubated with or without the indicated concentrations of tetrodotoxin in 1 mL of buffer that was sequentially removed and replaced every 4 minutes for 16 minutes, at which time veratridine was added to induce efflux and maintained throughout for 40 minutes. Experiments were terminated by adding 500 μL of 0.2% sodium dodecyl sulfate to solubilize cells, and radioactivity was determined along with the buffer samples by liquid scintillation counting in 10 mL Ready Safe (Beckman Instruments). Rate constants ($k$) for $[^3H]$Aspartate efflux at each time interval were calculated by means of the following equation:

$$k = \frac{\ln(A_t/A_0)}{(t_2-t_1)}$$

where $A_t$ and $A_0$ represent the total counts remaining in the dish at times $t_1$ and $t_2$, respectively. Data were also calculated as fractional loss of total disintegrations per minute released, to calculate percentage of total release after stimulation. Veratridine-stimulated release averaged 20% to 30% of total disintegrations per minute recovered. Basal release from untreated dishes was subtracted from stimulated release. Tetrodotoxin data are expressed as percentage of veratridine stimulated from seven separate titrations, using four different neuronal preparations that were prepared on separate days.

For measurements of intracellular calcium concentrations, $[^{45}Ca^{2+}]$, cells were grown on 14-mm² ACLAR plastic coverslips16 (Allied-Signal Engineered Plastics; available from Pro Physiologic, Inc.) and were centrifuged at 600 g for 10 minutes. For administration to gerbils, tetrodotoxin was dissolved in 0.9% saline and given as pretreatment in 5 μL intracerebroventricularly before ischemia as a single or replicate unilateral bolus. Vehicle-treated animals received 5 μL of saline, and sham animals were cannulated only. Coordinates for intracerebroventricular injection into the right lateral ventricle were as follows: 1 mm posterior to the bregma, 2 mm lateral to the midline, and 2 mm from the dural surface. Infusion was through a 27-gauge stainless steel needle during a period of 30 seconds. Although no behavioral side effects were noted in a previous study that used tetrodotoxin at approximately 0.64 ng⁴ and we noticed no effect from a 1- to 2-ng dose, our 5-ng dose did cause transient hyperactivity and rolling behavior in approximately half of the animals. There were no deaths among any group of gerbils. Seven days after occlusion, animals were killed, and formalin-perfused brain slices were prepared for histological examination and stained with 0.1% thionin; neuronal counts were determined by microscopic examination of coded slides to ensure unbiased observations. Morphologically normal neurons were counted over a 750-μm section of the right and left hippocampal CA1 regions, counts were averaged, and averages were recorded as a single value per animal. Data in text and figures are mean±SEM values. Statistical analysis was performed as indicated in the figure legends; significant difference was accepted at $P<.05$.

The rectal temperature of gerbils was monitored with an RET-3 rectal probe for mice; temporalis muscle temperature was measured using a TYPE MT-29/2 hypodermic needle microprobe (both from Physitemp Instruments, Inc.). Probes were coupled to an SCXI 1100 32-channel multiplexer amplifier and NIH MIO-16 data acquisition board in a Macintosh IIci computer running LabVIEW2 version 2.2 (all from National Instruments Corp).

Results

Unlike glutamate-mediated excitotoxicity,10,14,17,18 granule cell death induced by veratridine was not dependent on the cellular energy state and occurred in buffer containing glucose. A 20-minute pretreatment with tetrodotoxin dose-dependently prevented veratridine-mediated neuronal death with a protective concentration (PC⁵₀) of 22 nmol/L (n=6; Fig 1). Veratridine has been used to induce excitatory amino acid neurotransmitter release in cultured cerebellar...
granule cells, and we tested the ability of tetrodotoxin to inhibit this evoked release. [1H]Aspartate was used as a nonmetabolizable substrate of the Na+-dependent, Ca2+-independent glutamate transporter to label cytosolic reuptake pools (reference 8 and references therein). Data from a representative experiment are shown in Fig 2A. [1H]Aspartate release increased steeply from basal values after the addition of veratridine, peaked, and then declined with time. Tetrodotoxin dose-dependently inhibited the evoked release, with an inhibitory concentration (IC50) of 60 nmol/L (n = 7; Fig 2A and 2B). We showed that the evoked release of [1H]aspartate was through theNa+-dependent, Ca2+-independent glutamate transporter by repeating experiments under Na+-free or Ca2+-free conditions. Fig 3 demonstrates that the veratridine-stimulated release that we measured was totally dependent on Na+ and only 25% dependent on Ca2+

Cerebellar granule cell neuron preparations have an advantage over mixed cortical or hippocampal preparations in that they are approximately 95% pure neurons and are a more suitable model for studying changes in [Ca2+]i in large populations of central neurons. Measurements of [Ca2+]i, as an aggregate signal from approximately 400,000 neurons gave resting values of 96.5 ± 5.8 nmol/L (n = 54 from all historical data), similar to our and others' recently published values. Stimulation with 40 μmol/L veratridine caused an initial transient increase in [Ca2+]i (Fig 4A), lasting approximately 400,000 neurons gave resting values of 96.5 ± 5.8 nmol/L (n = 54 from all historical data), similar to our and others' recently published values.

Stimulation with 40 μmol/L veratridine caused an initial spike, then sustained increased levels of [Ca2+]i up to 495 ± 27 nmol/L (n = 54), a 5.1-fold increase over basal values (Fig 4A). Tetrodotoxin reverses the stimulated [Ca2+]i by a maximum of 80 ± 2.6% (Fig 4B); analysis of cumulative dose-response data yielded an IC50 of 63 nmol/L (n = 4 to 9; Fig 4B). Reversal of the veratridine-stimulated increase of [Ca2+]i was similar to what we previously observed for both carvedilol and (+)SKF 10 047 reversal of NMDA/glycine-induced increases of [Ca2+]i in these neurons, as well as what we recently reported for carvedilol reversal of veratridine-stimulated increases of [Ca2+]i. The increase of [Ca2+]i induced by veratridine was attributed to both voltage-sensitive Ca2+ channels and to reversal of the Na+-Ca2+ exchanger, since prior treatment with 2.5 μmol/L of the voltage-sensitive Ca2+ channel inhibitor nifedipine diminished the veratridine-stimulated release of 599 ± 36 nmol/L (n = 14) to 244 ± 14 nmol/L (n = 11) or 41% of the [Ca2+]i of the matched controls. Nifedipine did not affect the basal [Ca2+]i (96 ± 8 versus 110 ± 12 nmol/L for controls). The addition of 2.5 μmol/L MK-801 to the pretreatment with 2.5 μmol/L nifedipine diminished the veratridine-stimulated response to an average of 213 ± 14 nmol/L (n = 4) or 36% of the [Ca2+]i of the matched controls (Fig 5A). This slight added decrease was not statistically significant (one-way ANOVA), and therefore contribution of added Ca2+ influx via NMDA receptors seems unlikely. Veratridine depolarization equilibrated the membrane potential and blocked all voltage-sensitive Ca2+ channel activity since further addition of 50 mmol/L KCl did not provoke a depolarizing response (data not shown). However, blockade of Na+ channels by tetrodotoxin to effect Ca2+ efflux allowed subsequent Ca2+ influx through voltage-sensitive Ca2+ channels stimulated by 50 mmol/L KCl depolarization (Figs 4A and 5A). After addition of tetrodotoxin, there was a transient increase in [Ca2+]i, lasting approxi-
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before bilateral carotid artery occlusion provided a highly significant (P=.001 versus saline; n = 15) 91% neuronal protection (104±12 neurons versus 114±3 neurons for sham). However, 5 ng of tetrodotoxin administered intracerebroventricularly to conscious, nonischemic gerbils caused hypothermia of 3.5°C after 30 minutes (Fig 6D). Temperatures remained significantly depressed for 240 minutes (P<.001 to P=.019) except for the 270-minute time points annotated with asterisks.

At a lower dose of 1 ng, tetrodotoxin had no protective effect (32±8 neurons; n=11) and no hypothermic effect.

**Discussion**

Veratridine is toxic to cultured cells by acting as a channel activator at voltage-dependent Na+ channels, causing Na+ influx and ionic imbalance. A 20-minute pretreatment with tetrodotoxin dose-dependently prevented veratridine-mediated neuronal death by inhibiting Na+ channel activation. In the chick retina model of glutamate excitotoxicity, Zeevalk and Nicklas recently demonstrated that under conditions of severe metabolic stress, a combination of the Na+ channel blocker tetrodotoxin with the NMDA antagonist MK-801 afforded nearly full neuroprotection, completely inhibiting γ-aminobutyric acid and aspartate release and attenuating glutamate release by 70%. We and others have used tetrodotoxin to totally prevent veratridine-stimulated [3H]aspartate release from cerebellar granule cell neurons. Glutamate release from the brain is a major component of the "excitotoxic index," a measure of reperfusion injury after ischemic insult and a biochemical marker of selective vulnerability. Most ischemic glutamate release is thought to be nonvesicular in nature, arising from the reversal of the electrogenic, Na+-dependent, Ca2+-independent glutamate transporter, which is found in synaptosomes, glia, and in the selectively vulnerable hippocampus. During ischemia, excessive neuronal depolarizations will result in Na+ influx and ionic imbalances that will initiate the reversal of the glutamate transporter, causing release of cytosolic glutamate. As mentioned previously, sustained Na+ currents are often seen to contribute to oscillations in membrane potential independent of action potentials and could initiate ischemic damage. By modulating the activity of all neuronal Na+ channels, tetrodotoxin may prevent excessive depolarizations and serve to limit the excitotoxic glutamate release.

The ability of tetrodotoxin to inactivate Na+ channels and allow for Ca2+ extrusion and the reestablishment of ionic homeostasis points to the possibility of using Na+ channel modulators for postischemic therapy. The increase in [Ca2+]i seen after the addition of veratridine to cerebellar granule cells (Figs 4A and 5A) was only 40% because of reversal of the Na+-Ca2+ exchanger. The increase of [Ca2+]i seen in Fig 5B after the addition of tetrodotoxin is also evidence for reversal of the exchanger as high (millimolar) intracellular Na+ levels led to net Na+ efflux and resultant Ca2+ influx through reverse Na+-Ca2+ exchange. This type of reverse Na+-Ca2+ exchange has been implicated as a mechanism of anoxic damage in central nervous system white matter, where recovery of optic nerve compound action potentials was improved by pretreatment with bepridil and benzamil, nonspecific inhibitors of Na+-Ca2+ exchange. However, the Na+-Ca2+ exchange system in cultured cerebellar neurons has been recently shown to
provide a Ca\textsuperscript{2+} extrusion system that attenuates delayed glutamate excitotoxicity under conditions of sustained Ca\textsuperscript{2+} influx.\textsuperscript{27} We have also observed reversal of high [Ca\textsuperscript{2+}] in granule cell neurons after noncompetitive antagonist blockade of NMDA receptors.\textsuperscript{14,17} Similarly, hippocampal neurons exposed to Ca\textsuperscript{2+} overload by the ionophore A23187 or high KCl were killed; neurohybrid NCB-20 cells were resistant because of an apparently superior Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange system.\textsuperscript{28}

Inhibitors of reverse Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange may be of value only in a pretreatment paradigm\textsuperscript{5-26} before anoxic depolarization. The results of the ouabain experiment in Fig 5 are important in resolving this apparent contradiction. Without Na\textsuperscript{+} removal by the Na\textsuperscript{+}-K\textsuperscript{+} pump, the stoichiometry of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange (3:1) would be disrupted, with the net stoppage of Ca\textsuperscript{2+} efflux (Fig 5B) as intracellular Na\textsuperscript{+} concentrations remained high, favoring reverse exchange. Since the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange system is energy independent and only redistributes ions according to the directionality of the imposed gradients,\textsuperscript{29} a functional Na\textsuperscript{+}-K\textsuperscript{+} pump is therefore essential for Ca\textsuperscript{2+} removal after an ischemic episode. Because penumbral areas surrounding a cerebral infarct are still areas of low perfusion,\textsuperscript{3} it is possible to maintain enough ATP to preserve Na\textsuperscript{+}-K\textsuperscript{+} pump function. We may therefore speculate that selective inhibitors of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange would actually prove detrimental if administered after an ischemic event when neurons are seeking to repolarize. Tetrodotoxin and other Na\textsuperscript{+} channel blockers may therefore provide neuroprotection from ischemia-induced neuronal death by preserving cellular energy stores that are normally depleted by excessive ATP-dependent cation pumping as neurons struggle to repolarize under conditions of low blood flow.

The CA1 hippocampal protection that we saw in the gerbil global ischemia model closely follows that reported for the rat four-vessel occlusion model,\textsuperscript{4} in which 54% protection was achieved at a dose of 1 \mu mol/L or approximately 0.64 ng versus our dose of 5 to 6 ng that achieved 91% protection. However, the multiple dosing regimen of 2 ng (for a total of 6 ng) tetrodotoxin provided neuroprotection in the gerbil without being hypothermic, whereas the 5-ng bolus provided equivalent neuroprotection but provoked a lasting hypothermia. Hypothermia is a major consideration for neuro-
Fig 6. Graphs show that tetrodotoxin (TTX) is neuroprotective in gerbil global brain ischemia. TTX was administered intracerebroventriculally (i.c.v.) as 2 ng in 5 μL of saline at 3 hours, 2 hours, and 1 hour before bilateral carotid artery occlusion (A) or as 5 ng in 5 μL saline 30 minutes before occlusion (B). Seven days after 6 minutes' occlusion followed by reperfusion, animals were killed and brain slices were prepared for quantification of CA1 hippocampal neurons as described in “Materials and Methods." The number of gerbils used is highlighted in each bar. Data were analyzed by the Kruskal-Wallis procedure. TTX provided highly significant neuroprotection (P=.001) with each regimen. B and D show corresponding changes in temporalis muscle and rectal temperatures of nonischemic, conscious gerbils treated with the same neuroprotective regimens as in A and C. Although replicate additions of 2 ng TTX produced significant but quickly reversible hypothermia at only two time points, a 5-ng bolus of TTX produced significant temperature changes (P<.001 to P=.019) except for the 270-minute time points annotated with asterisks. Temperature data were analyzed by paired t tests.

protection in animal ischemia models, and the mechanisms by which various drug classes achieve this effect need to be understood. We and others have reported the hypothermic effects of glutamate receptor antagonists (Reference 32 and references therein). Since we have demonstrated neuroprotection by tetrodotoxin in a nonhypothermic regimen, we do not believe that hypothermia is the main neuroprotective mechanism. Therefore, it may be possible to develop novel Na⁺ channel blockers that can be administered as shown here for tetrodotoxin to provide neuroprotection without a hypothermic effect.

The mechanism of tetrodotoxin action for protection in gerbil global brain ischemia may be a combination of inhibition of Na⁺ influx, prevention of toxic Ca²⁺ overload, prevention of excitotoxic glutamate release, and preservation of cellular energy stores. The development of novel Na⁺ channel modulators that preferentially inhibit noninactivating cardiac Na⁺ channels also contribute to therapeutic development for cerebral ischemia and stroke, as suggested by the cerebroprotective agent BW619C89, which may interfere with Na⁺ fluxes during anoxic depolarizations. In cardiac Purkinje cells, a reduction of Na⁺ influx in depolarized cells occurred at inhibitor concentrations that did not interfere with normal cell conduction velocity. Therefore, low concentrations of a selective Na⁺ channel blocker would be most effective on the depolarized cells undergoing ischemic insult.

References


**Editorial Comment**

This article demonstrates the ability of tetrodotoxin to inhibit [3H]aspartate release in cultured rat cerebellar neurons and reduce cytotoxicity when exposed to veratrine. These findings demonstrate that elevated Na+ channel activity leads to enhanced neurotransmitter release and cell damage, an action that can be blocked by tetrodotoxin. Since veratrine exposure has been used as a model of ischemic damage in neurons, the authors examined the effect of tetrodotoxin in protecting ischemic neurons in vivo. Tetrodotoxin significantly reduced damage to CA1 hippocampal neurons in gerbils undergoing ischemic insult via bilateral carotid occlusion. Because tetrodotoxin administration (2 ng intracerebroventricularly) did not kill the animals or result in seizure activity, one can speculate that ischemic neurons become "sensitized" to the Na+ channel blocking actions of tetrodotoxin, leaving "normal" neuronal function relatively unaffected. It is also possible that the dose of tetrodotoxin used was not sufficient to completely block Na+ channel activity but enough to reduce activity sufficiently to reduce energy demand. While these studies suggest that one of the mechanisms involved in ischemic neuronal damage may be the inability of cells to maintain a normal Na+ gradient, it is also possible to speculate that a general reduction in neural activity protects against ischemic damage by reducing energy demand. Regardless of the mechanism, the study suggests a therapeutic potential for the treatment of ischemic insult by selective Na+ channel blockade.

David R. Harder, PhD, Guest Editor Cardiovascular Research Center Medical College of Wisconsin Milwaukee, Wis
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P G Lysko, C L Webb, T L Yue, J L Gu and G Feuerstein

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