Transtympanic tetrodotoxin alters the VOR and Fos labeling in the vestibular complex

Dale W. Saxon,[CA] John H. Anderson[1] and Alvin J. Beitz[2]

Department of Anatomy and Cell Biology, Indiana University School of Medicine, Evansville Center for Medical Education, 8600 University Blvd., Evansville, IN 47712; [2]Department of Otolaryngology, University of Minnesota, Minneapolis, MN 55455; [3]Department of Veterinary Pathobiology, University of Minnesota, 1988 Fitch Ave., St. Paul, MN 55108, USA

[CA]Corresponding Author

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The sodium channel blocker tetrodotoxin (TTX) is an effective tool for blockade of action potentials. Unilateral transtympanic administration of 3 mM TTX produced behavioral symptoms similar to those following unilateral peripheral vestibular ablation. Complete resolution of visible symptoms occurred between 48 and 72 h post-TTX. Eye-coil recordings indicated a spontaneous nystagmus and a decrease in the VOR in TTX-treated animals. Neuronal activity in the central vestibular complex (VC), as monitored with Fos immunocytochemistry, revealed an asymmetric pattern of Fos labeling in the medial, inferior and superior vestibular nuclei and the prepositus hypoglossal nucleus. Although the spatio-temporal pattern of Fos labeling was consistent and reproducible at each time-point, changes were noted among time-points. Transient blockade with TTX may be useful for studying the central vestibular response to recurrent or episodic vestibular disruption in the intact system. *NeuroReport* 12:3051–3055 © 2001 Lippincott Williams & Wilkins.

Key words: Inner ear; Nystagmus; Tetrodotoxin; Vestibular

INTRODUCTION

As one of the oldest and most conserved sensory systems in vertebrates, the vestibular system plays a pivotal role in many of the most rudimentary daily activities of animals and humans. In its capacity to transduce, integrate and pass along essential information relating to the maintenance of balance and equilibrium, disruption or damage to the vestibular system results in debilitating and characteristic symptoms. On the other hand, following permanent damage to the peripheral vestibular apparatus the system as a whole has been shown to have remarkable plasticity as the static and dynamic symptoms resolve to varying degrees in a process referred to as vestibular compensation [1–7]. Investigation into the underlying mechanisms of vestibular function/dysfunction has been prompted by the involvement of the vestibular system in a number of clinically relevant pathological conditions including Menière's disease. To date our understanding of the function/dysfunction of the vestibular system has relied almost exclusively on models that involve partial or total ablative methodologies. Although it is indisputable that such studies provide useful information about the vestibular system, the application of lesion or permanent ablative techniques in studies of the functional organization of the brain has well known limitations [8]. This issue is particularly poignant when one considers that a clinical condition such as Menière's disease presents with acute but episodic attacks of vertigo [9], a feature that cannot be recreated using a permanent ablation model.

Tetrodotoxin (TTX) is a potent blocker of voltage-dependent sodium channels in excitable cells and has been used successfully in a number of studies in the CNS and PNS to produce a fully reversible blockade of action potentials [8,10–13]. The present study reports on the transtympanic administration of TTX in the rat as a transient and non-invasive method for producing functional blockade of the peripheral vestibular apparatus. This novel model offers a potential opportunity to study central vestibular responses following recurrent or episodic vestibular events in the intact vestibular system.

MATERIALS AND METHODS

A total of 33 male Sprague–Dawley rats (250–300 g) were used in the present study. Twenty-one rats (3/group) received unilateral transtympanic injections of TTX (75 µl; 3 mM) in PBS and were perfusion-fixed at various time-points (2, 12, 24, 48, 72 and 96 h) post-TTX injection. Subjects were briefly (< 5 min) anesthetized with Isoflurane in a closed chamber. TTX loaded into a 100 µl Hamilton syringe was delivered to the middle ear cavity by passing the needle through the tympanic membrane. Prior to perfusion with 4% paraformaldehyde in PBS (pH 7.3) subjects were deeply anesthetized with sodium pentobarbital (70–80 mg/kg, i.p.). Following fixation the brains were excised and cryoprotected in a 25% sucrose solution overnight.

Frozen brain sections (35 µm) were processed for Fos immunocytochemistry (Santa Cruz Biotechnology Inc.,

1:8000 dilution primary antibody) using standard immunocytochemical methods with DAB as the chromogen. Brain sections were mounted on gel-coated slides, coverslipped and viewed on an Olympus BX50 microscope. Images were captured using a Spot RT digital camera (Diagnostic Instruments Inc.) interfaced with a G3 Power Mac.

The temporal bones of two skulls (survival time 7 days) were placed in a decalcifying solution of trifluoro-acetic acid (5%) for 5–7 days. Sections (8 μm) through the inner ear were cut from decalcified bones embedded in paraffin and these were subsequently stained with hematoxylin and eosin. Six rats (survival time 2 h) were divided into two control groups, group 1 received a transtympanic injection of 75 μl PBS ($n = 3$) while group 2 received no treatment other than being anesthetized. The three remaining rats received three injections into the same ear with 7 days between each injection and were perfused 2 h after the last injection.

For Fos immunocytochemistry, non-specific reactions were evaluated by omitting incubation either in the primary or secondary antibody. This procedure resulted in no positive Fos staining.

The horizontal vestibulo-ocular reflex (VOR) of three animals was recorded prior to and 2 h after unilateral TTX injection into the middle ear. The animals were fully alert for all recordings but immobilized with a custom-fitted meshwork that covered the body and head. In addition there was a small bit bar that helped to fix the head to the platform to which the meshwork was attached. The platform was mounted on a turntable that could be rotated in the horizontal plane. To test the VOR animals were rotated sinusoidally in complete darkness at several frequencies between 0.05 and 0.4 Hz. The peak velocity of the rotation was 115°/s. The magnetic search coil technique was used to record the eye movement. A sine wave was fitted to the slow phase component of the eye velocity. The quick phases of the nystagmus were removed before the fitting and separate calculations were made for rotation to the right and left so that any asymmetry could be quantified. The spontaneous nystagmus that was present after TTX injection contributed a small DC component to the eye velocity, which was subtracted and did not affect the peak-to-peak sine wave measurements.

All experimental procedures were approved by the Animal Care and Use Committees at Indiana University and the University of Minnesota and carried out in line with the proposals of the IASP Committee for Research and Ethical Issues such that the number of animals used for these studies was minimized.

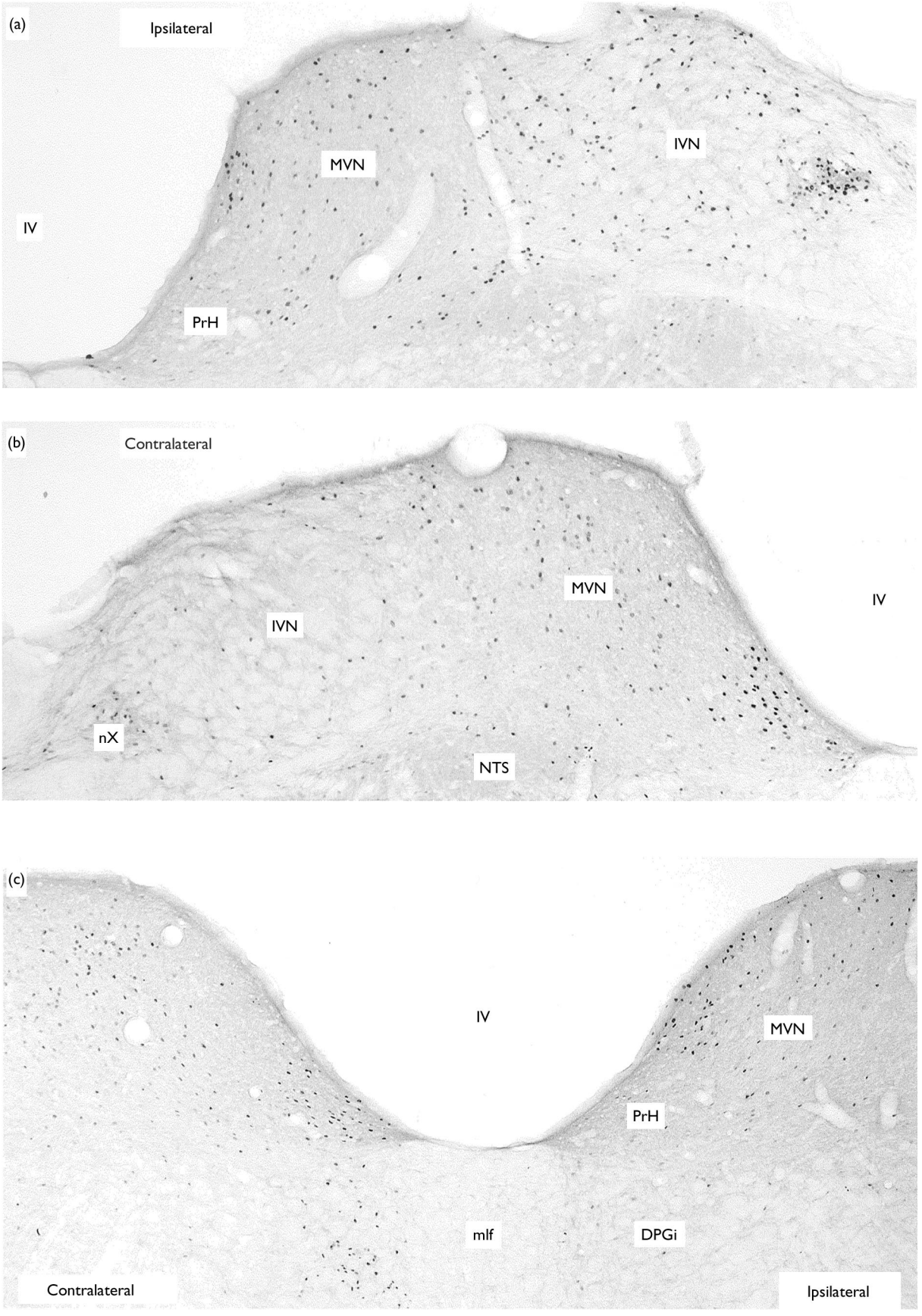
RESULTS

Following rapid recovery from anesthesia (< 5 min) behavioral activity was monitored every 15 min for the first 3 h, every hour for the next 9 h and then every 12 h to 96 h. Immediately after recovery from anesthesia subjects resumed normal exploratory behavior for a short time but within 15–30 min post-TTX their movement became shaky and unstable. They typically maintained a static posture with the front limbs rigidly splayed to the side and the head low to the ground. Audible grinding of the teeth and slight head bobbing was not uncommon. By 45–60 min inactivity was predominant and even when prodded subjects were reluctant to move, occasionally vocalizing instead of moving. Head tilt and neck torsion in the direction of TTX administration was evident between 30–60 min post-TTX and persisted to varying degrees for > 48 h. Spontaneous barrel roll behavior was observed in only one animal although all subjects (2–24 h) when picked up by the tail rotated violently along their long axis in the direction of TTX treatment. At 2 h post-TTX subjects placed in a novel environment (laboratory table) tended to remain immobile but when prodded to move preferred backward locomotion, often while turning in the direction of TTX treatment. By 24 h subjects were mobile but an obvious head tilt and unsteady gait were evident. Similar observations to those at 24 h were made for animals at 48 h post-TTX, although picking these animals up by the tail did not always induce spinning behavior. Between 48 and 72 h visible behavioral signs of vestibular disruption were largely resolved except for a slight head tilt in one subject. An interesting note was that almost all the animals perfused at or before 48 h post-TTX began to spontaneously barrel roll in the direction of the TTX treatment when deeply anesthetized prior to perfusion. By 96 h post-TTX and in the control groups there were no observable signs of vestibular disturbance.

Subjects displayed a tonic eye deviation toward the TTX-treated side as well as a spontaneous oculomotor nystagmus with the slow phase toward the TTX side and the fast phase toward the contralateral side. Figure 2 shows the effect of TTX on the VOR. There was a significant reduction in the amplitude of the VOR and an asymmetry. The response during rotation to the TTX-injected ear was less than rotation to the normal ear at all frequencies tested. All three animals that had VOR recording showed this behavior. An asymmetry score was calculated as follows. After subtracting the eye velocity due to a spontaneous nystagmus, the difference between the gains calculated separately for rotation to the right (TTX side) and to the left (normal side) was divided by the average of the gains. The mean scores for all animals after TTX injection were 0.214, 0.166, and 0.349 at frequencies of 0.05, 0.1, and 0.2 Hz. The average across all frequencies was 0.264 ± 0.154 and was significantly different from the pre-injection value (0.018 ± 0.013), based on the Kolmogorov-Smirnov test ($p < 0.02$).

Microscopic observations on hematoxylin and eosin

Fig. 1. Digital photomicrographs of sections taken from an animal receiving a unilateral transtympanic injection of TTX (survival time of 24 hours). (**a**,**b**) are from the same section and illustrate the asymmetric pattern of Fos labeling between the two sides of the caudal portion of the VC, prepositus hypoglossal nuclei (PrH) and nX (nucleus X). Note the prominent Fos labeling in the contralateral prepositus hypoglossal nucleus and ipsilateral nX. (**c**) Fos labeling in the rostral MVN and prepositus hypoglossal nuclei. Note the distinct absence of stained nuclei in the dorsomedial region of the contralateral MVN that are evident in the ipsilateral MVN. Also note the presence of stained nuclei in the contralateral DPGi along the medial longitudinal fasciculus (mfl). Labeling shown here 24 h post-TTX is similar in distribution to that observed at earlier time-points although the intensity of Fos staining and the relative numbers of stained nuclei tend to be greater at earlier time-points. The inferior vestibular nucleus is denoted by the abbreviation IVN.


(a)
Ipsilateral
MVN
IVN
IV
PrH
(b)
Contralateral
MVN
IV
IVN
nX
NTS
(c)
IV
MVN
PrH
mlf
DPGi
Contralateral
Ipsilateral

stained sections (not shown) containing the ampulla of the horizontal canal and macula of the utricle revealed a normal morphology for these structures 7 days post-TTX treatment.

Perikarya containing Fos-positive nuclei were found at basal levels in numerous regions of the brainstem including the VC. The presence of small numbers of Fos positive nuclei in the VC in particular the MVN is consistent with our own observations and with those previously reported for the rat brain [3,14–16]. However following unilateral treatment with TTX, Fos staining in the brain stem particularly in the VC was markedly changed relative to control levels. In addition to increased numbers of positively stained nuclei there was a clear asymmetry in the pattern of Fos labeling between the ipsilateral (i.e. TTX side) and the contralateral MVN (Fig. 1). Asymmetry in Fos labeling was also observed between the ipsilateral and contralateral prepositus hypoglossal nuclei, inferior and to a lesser degree the superior vestibular nuclei. Of the six time points tested asymmetry in Fos labeling in the VC was most prominent 2 h post-TTX but was readily observed at 12, 24 (Fig. 1) and 48 h. By 72 h post-TTX the relative numbers and intensity of Fos labeling in the VC and prepositus hypoglossal nuclei was greatly diminished. At 96 h Fos labeling in the inferior and superior vestibular nuclei had returned to control levels although asymmetry in Fos labeling in the MVN and prepositus hypoglossal nuclei was still present albeit greatly attenuated.

Other parts of the brain stem not described in this paper but which nevertheless displayed spatio-temporal changes in Fos labeling included the inferior olive, nucleus tractus solitarius, trapezoid body, nucleus X and Y, and the molecular and granule cell layers of the cerebellum.

Rats receiving successive unilateral injections of TTX displayed a similar pattern of behavioral symptoms as those observed following a single unilateral injection. In addition the pattern of Fos labeling (at 2 h) in the VC following successive injections was comparable to that reported herein for the single unilateral TTX treatment.

DISCUSSION

In the present study we have modified the methodologies outlined in two previous reports [10,17] to develop a technique whereby TTX deposited in the tympanic cavity diffuses across the round and oval windows to access the peripheral vestibular apparatus. This model of vestibular disruption offers several unique features for the study of the vestibular system not permitted by traditional ablation models. Perhaps most notable of these is the potential to study central vestibular changes following transient disruption in the intact system, removing potentially confounding factors such as peripheral axonal reactions and central glial reactions which must be a consideration in any ablation model. In addition preliminary results (not shown) indicate that repeat administration of TTX to the same side following complete recovery induces similar behavioral symptoms as those reported in the present study making investigation into episodic vestibular disruption a distinct possibility. In light of these novel features the TTX model is likely to be a useful tool in the ongoing investigation into vestibular function/dysfunction.

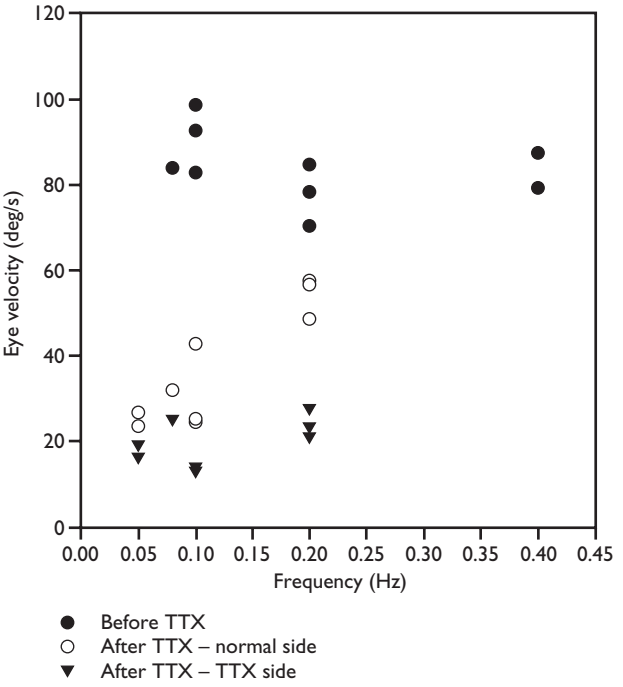


Fig. 2. Horizontal VOR before and after TTX injection. The amplitude of the sine wave fitted to the eye velocity is plotted. Note that after TTX there was a decrease in the VOR for rotations in both directions, but there was a greater decrease for rotation to the injected side.

It was noted that awake animals rarely showed spontaneous barrel roll activity but that this activity was evident during the onset of terminal anesthesia. The authors suspect that the rare nature of spontaneous barrel roll is in part the result of the rapid recovery from anesthesia. Rapid recovery permits each animal to be conscious and able to respond with behavioral activities and perhaps activate some central mechanism(s) that help dampen and/or adapt to the onset of vestibular blockade by TTX. It seems clear from the aforementioned observations that barrel roll activity is possible but only as a consequence of the loss of some other input. For instance picking the animal up by the tail removes proprioceptive cues that have previously been suggested as important sensory inputs for stabilization and compensation in the absence of vestibular input [2,5,6,18,19]. Barbiturate anesthesia would be expected to depress many neural systems including the vestibular, although sensitivity between systems is unlikely to be entirely uniform. This being the case any adaptive mechanism(s) put in place in response to TTX blockade could be disrupted by barbiturate anesthesia and thus allows the barrel roll activity to develop.

Two important technical issues can be raised regarding the use of the TTX methodology. The first relates to the possibility that TTX causes permanent damage to the peripheral vestibular apparatus and the second that TTX is acting systemically. The authors are confident that there is no permanent damage to the vestibular apparatus based both on the histological observations of the inner ear and on the ability of TTX reapplication to the same ear to replicate the behavioral symptoms observed prior to total

recovery. Although the methodology in every instance resulted in symptoms of vestibular disruption, some variability in the time course of onset did occur and can probably be attributed to either the proximity of the deposited TTX to the round and/or oval window or to the time for sufficient TTX to diffuse into the inner ear. Diffusion could be affected by a number of factors including the thickness of the tissue covering the windows as well as trauma-induced bleeding [20]. Although vascular, puncture of the tympanic membrane by the needle never resulted in substantial bleeding, there is always the possibility that blood from the tympanic membrane or inadvertent damage to the middle ear cavity could dilute the TTX and/or interfere with its diffusion into the inner ear. TTX could potentially gain systemic access via the circulation, inner ear and/or eustachian tube. However there are two lines of evidence that argue against the likelihood that TTX has substantial action via any of these systemic routes, the first being the asymmetry in the behavioral effects and the second being the consistent asymmetry in the anatomical results.

From a behavioral standpoint unilateral application of TTX resulted in the rapid onset of symptoms consistent with those reported following unilateral vestibular ablation or VIII nerve neurectomy [2–6,20,21]. However unlike ablation models which typically require recovery times of 1–2 h following surgery before behavioral observation commence, the TTX model with its rapid recovery (<5 min) offers the opportunity to observe early behavioral events in a fully alert subject. For example, the gradual cessation of movement in the first hour following TTX treatment and the concomitant postural changes such as lowering the head and flattening the trunk with the front legs splayed apart are not evident in the surgical models. Such responses are not unlike those one might expect to see in a human experiencing the onset of vestibular disruption, i.e. effort to stabilize their body, perhaps laying down and remaining immobile. Whether or not there are correlative neuroanatomic changes that can be detected prior to 2 h post-TTX remains to be investigated.

Immunocytochemistry for Fos protein is now regarded as a reliable method for the cellular detection of neuronal populations activated by a variety of stimuli [3,4,15,22]. Mapping of Fos protein has been employed in a number of studies to characterize neuronal activity in the central vestibular complex [1,3,4,7,15,16,23,25] and up-regulation of Fos in the brain is in some instances thought to reflect the activation of specific neural pathways [25]. With this in mind the general asymmetry in the patterns of Fos expression in the VC is likely to reflect differences in the pathways activated on the two sides of the VC. Furthermore these activated pathways appear at least in part to temporally correlate with behavioral observations and may or may not correspond to the same pathways activated following unilateral ablation of the peripheral vestibular apparatus. In a recent study, behavioral compensation was altered following hemilabyrinthectomy by application of antisense probes directed at c-fos mRNA which blocked Fos protein expression [7]. Based on the extent of Fos expression in the vestibular nuclei following TTX treatment it is likely that antisense treatment would also affect any Fos related processes associated with TTX blockade of the VIII cranial nerve. Immediate early gene proteins like Fos are transcription factor proteins which regulate gene expression within cells and they have been linked to the activation of late response genes which are believed to be part of a cascade of events that can lead to phenotypic changes in response to environmental stimuli [24]. Other immediate early genes have been investigated and implicated in vestibular compensation following unilateral vestibular ablation [5]. Further evaluation of the patterns of immediate early genes and/or their protein products following TTX treatment could assess the similarities and differences between the response of the central VC to permanent unilateral ablation and transient (functional) blockade in the intact system.

CONCLUSION

The current study provides behavioral, anatomical and physiological evidence to support the idea that transtympanic injection of TTX is a reliable methodology for producing transient disruption of the intact vestibular system. Use of the TTX model to investigate and identify specific neuronal populations activated in the VC during transient and episodic disruption may provide insight into potential targets for treatment of human and animal syndromes in part characterized by debilitating episodic vestibular disturbances.

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