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Research report

Episodic blockade of cranial nerve VIII provokes asymmetric changes in lobule X of the rat

Dale W. Saxon*, Gary White

Department of Anatomy and Cell Biology, Indiana University School of Medicine, Evansville Center for Medical Education, 8600 University Blvd., Evansville, IN 47712, USA

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Abstract

Although debilitating syndromes like Ménière's disease are in part characterized by recurrent or episodic vestibular disturbance the study of episodic vestibular disruption has only recently been possible with the introduction of a new model utilizing tetrodotoxin (TTX). In the present study, serial unilateral transtympanic administration of TTX produced behavioral symptoms indicative of transient vestibular disruption and novel patterns of Fos activity in the brainstem and cerebellum. Following two or three serial injections of TTX and a final survival time of 2 h, Fos immunocytochemistry revealed a distinct pattern of labeling in the brainstem that differed temporally from that observed following a single unilateral TTX injection. Specifically there was protracted expression of Fos in the β subdivision of the inferior olive (IO) on the side ipsilateral to TTX treatment. In the cerebellum, the hallmark of episodic vestibular blockade was an asymmetric pattern of Fos labeling that involved all three layers of the cortex. In particular, there was prominent Fos labeling of Purkinje cells in the contra-TTX half of lobule X. In view of the fact that Fos labeling is not found in Purkinje cells following a single transient event or following peripheral vestibular ablation, it is suggested that Fos expression in Purkinje cells is a unique feature of episodic vestibular disruption and may represent a novel plastic response by a select population of Purkinje cells to episodic functional deafferentation.

Keywords: Tetrodotoxin; Vestibular; Cerebellum; Purkinje cell; Labyrinthectomy; c-*fos*

1. Introduction

Although it was long held that the adult brain lacked any significant structural and functional plasticity, it is now well accepted that such adaptive processes do occur throughout life and that the possibility exists to modify and/or enhance these processes. Neuroplasticity includes changes at all levels of neuronal organization and includes such things as axon and dendritic remodeling, cell death and a host of molecular changes. Such possibilities have spawned numerous investigative efforts directed at understanding the sequence of events underlying plasticity in various adult systems. The auditory system with its prominent clinical significance has attracted a great deal of interest particularly related to understanding plastic phenomena provoked by changes in input activity to that system. At the cellular level, intracochlear stimulation has been shown to provoke molecular processes and gene activity related to plasticity [25,57,58]. In the auditory system, it has been proposed that stimulation of the sensory epithelium initiates signal cascades at two different levels into the CNS, a fast (or ionic) and a slow (or molecular) response channel [24]. The fast response channel consists of electrical signals passed between neurons in an ascending chain in response to adequate stimulation of a sensory end-organ. By contrast, the slow response channel involves chemical changes inside neurons that may be related to the particular patterns of activity experienced and require minutes or much longer to develop. Further, it was suggested that, as specific patterns of activity present in the fast channel, these will trigger a slow response, which in turn will specify response characteristics of neurons with respect to the fast channel [24].

The vestibular sensory system is closely related to the auditory system and has also attracted considerable attention with respect to the process of vestibular compensation.

* Corresponding author: Tel.: +1-812-464-1835; fax: +1-812-465-1184.
E-mail address: dsaxon@IUPUI.edu (D.W. Saxon).

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Vestibular compensation is a form of neural plasticity that occurs following permanent peripheral vestibular ablation [12,15,56]. From a clinical perspective, the vestibular system also has significance due to syndromes like Ménière's disease that are in part characterized by recurrent bouts of debilitating vestibular dysfunction (see Ref. [3]). Relatively little is know about the brain's response(s) to disruptive episodic events in the intact system. Recently, a series of publications [52,53,55] utilizing the transtympanic administration of tetrodotoxin (TTX) to produce transient blockade of neurotransmission in the VIIIth cranial nerve has presented an opportunity to look at episodic vestibular disruption. In light of the fact that the aforementioned "slow response" can be activated by lesion or permanent disruption of the sensory apparatus and that patterns of activity can also induce a slow response [24], it is of interest to see whether or not episodic functional deafferentation of the vestibular component of the VIIIth cranial nerve will precipitate detectable changes that could represent a slow response to a pattern of sensory deprivation. Fos the immediate early gene product of c-*fos* is among a number of molecules that have been implicated in processes related to plasticity and would be considered a part of a slow response [24,26]. Fos expression has been used to map pathways in numerous regions of the brain in response to a variety of physiologic and pathophysiologic stimuli [9,11,14,16,17,22,27,28,30–33,42,50]. In the vestibular system, the induction of Fos in specific neuronal populations has provided information about the characteristic patterns of activity associated with unilateral peripheral vestibular ablation [11,31,33,37–40] and transient vestibular disruption [52,53,55]. The present qualitative study utilizes the transtympanic TTX methodology in combination with Fos expression to investigate whether or not episodic vestibular disruption induces a different pattern of Fos activity in the brainstem and cerebellum versus that following a single TTX treatment. In other words, does previous experience with a defined stimulus (in this case transient blockade of neurotransmission by TTX) alter the response of the brain relative to a single novel event?

2. Materials and methods

A total of 15 male rats (Sprague–Dawley, 300–375 g) were used in the study. All rats regardless of the treatment regimen were briefly (< 5 min) anesthetized with Isoflurane. Experimental animals received either two or three transtympanic injections of TTX (75 μl of 3 mM TTX in PBS, pH 5.0), on the same side, each injection 2, 7 or 14 days apart. In the morning, rats were transported a short distance in opaque plastic cages to the lab where they were injected no more than 30 min after arrival in the lab. Recovery in the same cages occurred in the lab at which time they were returned to the animal facility. All animals were handled in a similar manner. Details of the injection protocol have been published previously [55]. Three SD rats served as controls and each received three transtympanic injections of an equal volume of vehicle, 7 days apart.

Subjects were deeply anesthetized (75 mg/kg sodium pentobarbital) and transcardially perfused with 4% paraformaldehyde in PBS (pH 7.3) 2 h following the final TTX or PBS injection. Following fixation, the brains were removed and cryoprotected in a 25% sucrose solution overnight. Prior to sectioning, a cut was made along the long axis of the ventral aspect of the brainstem on the side contralateral to the TTX injection and a three-edged needle passed through the cerebellum on the side ipsilateral to TTX treatment to ensure accurate identification once the sections were ready for viewing. Coronal or sagittal sections (35–40 μm thick) through the brainstem and cerebellum were cut on a sliding sledge microtome fitted with a freezing stage. In most cases, sections were collected from the level of the motor decussation rostral to the level of the parabrachial nucleus. Free-floating sections were processed for Fos immunocytochemistry (Santa Cruz Biotechnology, rabbit polyclonal Lot# D100, 1:8000 dilution primary antibody) using standard immunocytochemical methods with DAB as the chromogen. In order to maximize Fos labeling and limit background staining, incubation in the primary antibody was for 48 h at 4 °C. Brain sections were mounted on precleaned Superfrost Plus slides (VWR, cat. # 48311-703),

Table 1
A general comparison between Fos expression in the brainstem and cerebellum following a single unilateral TTX treatment and serial unilateral PBS and TTX treatments

	Single TTX (2 h)		3 × PBS (7 days, 2 h)		3 × TTX (7 days, 2 h)	
	Ipsi-TTX	Contra-TTX	Ipsi-TTX	Contra-TTX	Ipsi-TTX	Contra-TTX
IO	0	+++	0	0	++	+++
MVN	+++ (asymmetric pattern)	+++	+	+	+++ (pattern same as single TTX injection)	+++
g	+++	++	+	+	+++	++
m	+++	+	0	0	+++	+
PC	0	0	0	0	0	+++

Note that it is only following serial TTX treatment that induced Fos expression is found in the ipsi-TTX IO and in Purkinje cells of the contra-TTX half of lobule X. Also note that, with the same final survival time, a single or serial TTX treatment results in the same distinctly asymmetric pattern of Fos labeling in the MVN and in the granular (g) and molecular (m) layers of lobule X. 0 = no Fos, + = controls levels, ++ = moderate and +++ = high.

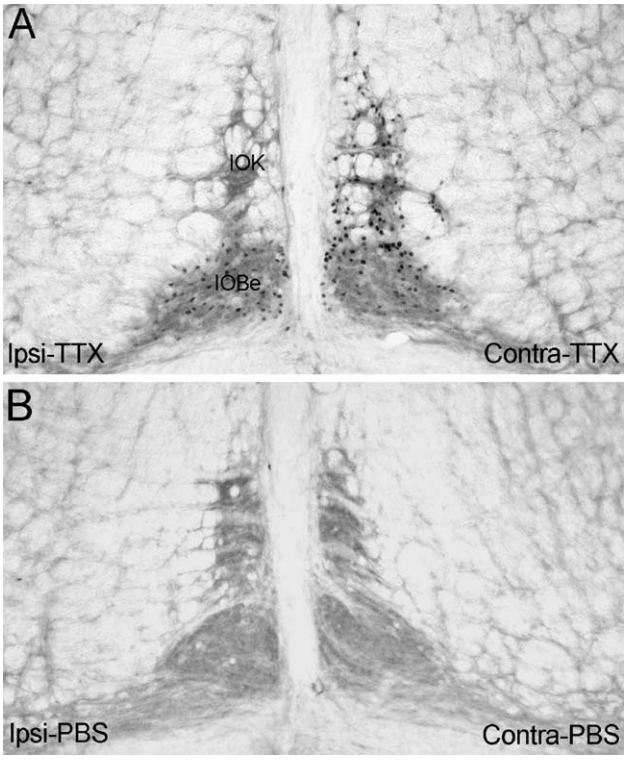

Fig. 1. Digital micrographs of transverse sections through the caudal IO. (A) Bilateral Fos labeling in the β subnucleus (IOBe) in an animal receiving 2 transtympanic injections of TTX on the same side, 7 days apart and having a final survival time of 2 h. Also note the distinctly unilateral Fos labeling in the contra-TTX cap of Kooy (IOK). (B) Absence of Fos labeling in the IO in a section taken from a control animal receiving three transtympanic injections of vehicle on the same side, 7 days apart and having a final survival time of 2 h.

cover-slipped and viewed on an Olympus BX50 microscope. Images were captured using a Spot RT digital camera (Diagnostic Instruments) interfaced with a G3 Power Mac.

For Fos immunocytochemistry, non-specific reactions were evaluated by omitting incubation either in the primary or secondary antibody. Omitting either the primary or secondary antibody resulted in no positive Fos staining. All treatments and experimental procedures were in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 80-23) and followed protocols approved by the Animal Care and Use Committee at Indiana University.

3. Results

As in previous publications [52,53,55] behavioral observations provided a guide for conformation of unilateral vestibular disturbance. Following each injection, subjects were monitored at 15-min intervals, out to 2 h for gross behavioral symptoms. Head tilt in the direction of TTX treatment was the principal unprovoked indicator of unilateral vestibular disturbance. Previous experience in the lab revealed that there is a tendency on the part of TTX treated rats to remain immobile in the first few hours [53] and thus it is useful to elevate (30–40 mm) the rat off the floor of the cage by grasping it by the base of the tail. The removal of proprioceptive cues from the limbs elicits more dramatic vestibular symptoms such as neck and trunk torsion and barrel roll behavior toward the side of TTX treatment. At 15 min following the first TTX injection, all animals displayed head tilt in the direction of TTX treatment. Subsequent serial injections also produced head tilt. A brief elevation by the tail immediately prior to terminal anesthesia produced neck and trunk torsion. However, in contrast to a single TTX injection where tail lift in virtually every instance results in pronounced twisting and even violent barrel rolling along the long axis of the body, serially injected animals

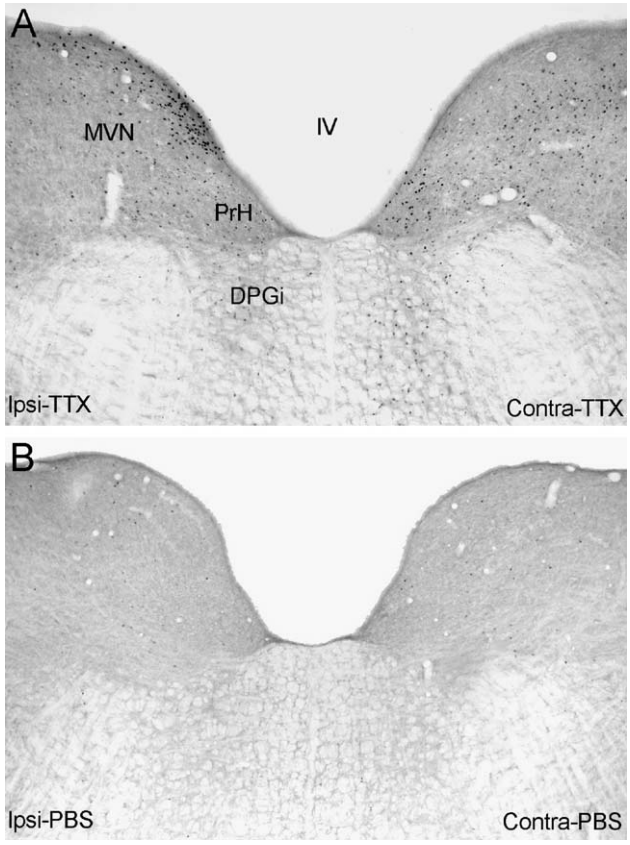

Fig. 2. Digital micrographs of transverse sections through the medial vestibular nucleus (MVN). (A) Fos labeling in an animal receiving two transtympanic injections of TTX on the same side, 7 days apart and having a final survival time of 2 h. Note the distinctly asymmetric distribution of Fos characteristic of unilateral functional deafferentation of the VIIIth nerve by TTX. Note the concentration of Fos positive profiles in the dorsomedial aspect of the ipsi-TTX MVN and paucity of Fos in the corresponding site contralaterally and the prominent labeling in the remainder of the contra-TTX MVN relative to the same location ipsilateral to TTX treatment. Incidentally, the distribution of labeling in the MVN is similar regardless of the treatment being singular or multiple. (B) Fos labeling in an animal receiving three transtympanic injections of vehicle on the same side, 7 days apart and having a final survival time of 2 h. PrH = prepositus hypoglossi nucleus, DPGi = dorsal paragigantocellular nucleus.

did not displayed such dramatic symptoms. This was particularly true of those rats receiving three TTX injections. At no time did any of the control animals display symptoms characteristic of unilateral vestibular disturbance.

Fos labeling was exclusively localized to the nuclei of cells. The distribution of Fos in the brainstem and cerebellum of control animals (see Table 1) was symmetric with regard to the two sides of the brain and was similar to that previously reported for animals receiving no treatment [9,21]. In brief and with regard to those regions relevant to the current study, there was virtually no Fos found in any of the subdivisions of the inferior olive (IO) (Fig. 1B). There were small numbers of stained nuclei found throughout the vestibular complex although stained profiles were most prominent in the medial vestibular nucleus (MVN) (Fig. 2B). In the cerebellum, Fos labeling was limited to the granular cell layer and consisted of scattered stained nuclei and small clusters of faint to moderately stained nuclei (Fig. 3A–C).

Fos labeling in the IO, MVN and in lobule X of the cerebellar cortex was significantly altered by serial TTX injections. In subjects with the 7-day injection interval and

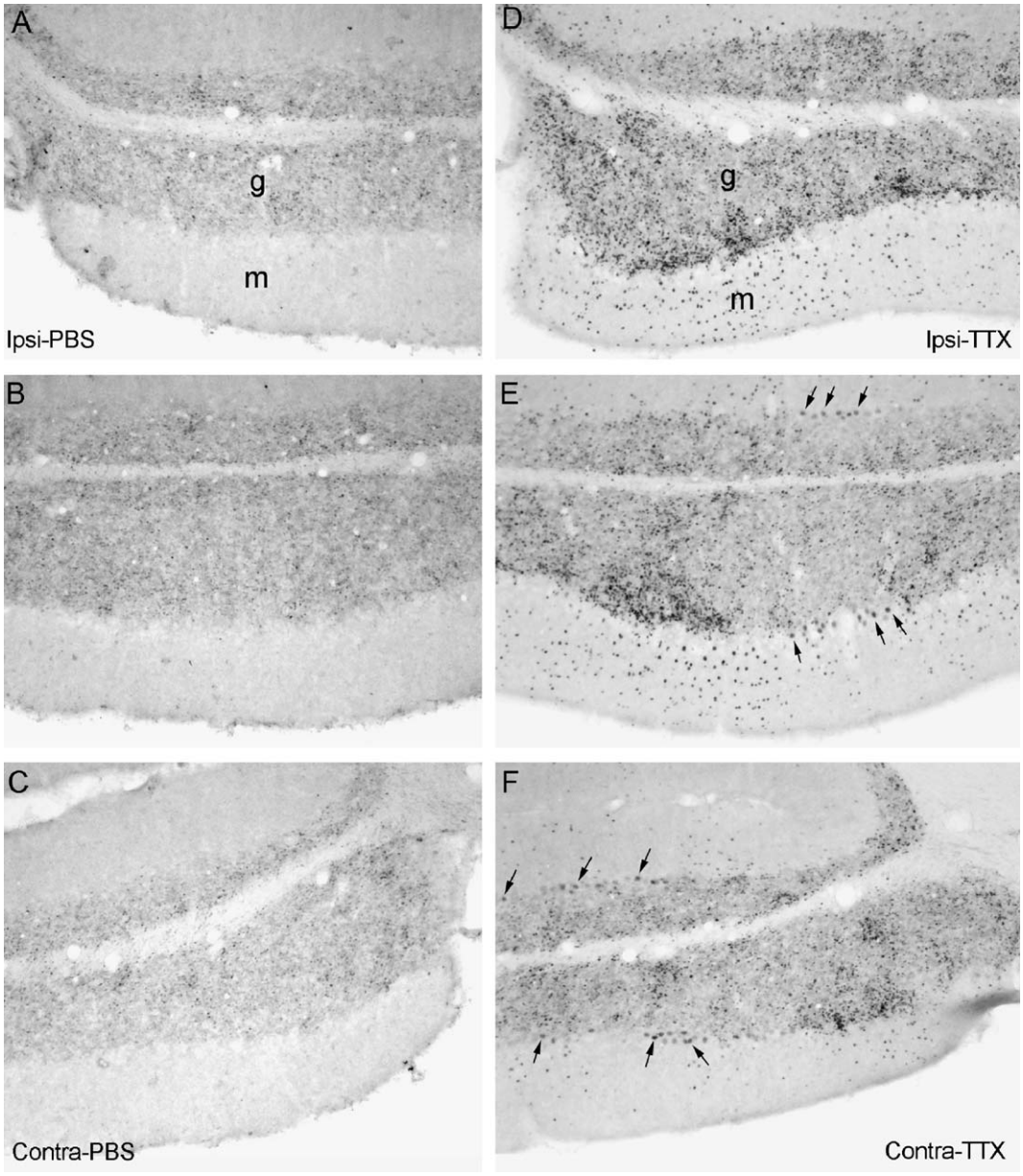

Fig. 3. Digital micrographs of sections through lobule X taken from an animal receiving three transtympanic injections of vehicle on the same side, 7 days apart and having a final survival time of 2 h (A–C), and another animal receiving three TTX injections, 14 days apart and having a final survival time of 2 h (D–F). In A–C, note the sparse Fos labeling in the granular (g) layer and the absence of Fos expression in the molecular (m) and PC layers. (D–F) Prominent Fos labeling in the ipsi-TTX granular and molecular layers (D) and Fos-labeled PCs (arrows) in the contra-TTX half of lobule X (E and F). Note that the pattern of Fos labeling in the 14- and 7-day (Fig. 4) animals are very similar.

2-h final survival time, some subnuclei of the IO displayed bilateral Fos expression. Caudally, there were small numbers of faintly labeled nuclei in the IOA and IOB on both sides of the brainstem. In addition, there was bilateral labeling in the IOBe (Fig. 1A) although the intensity of the Fos staining was considerably more uniform and intense than that observed in the IOA and IOB. Contralateral to the TTX injections prominent Fos labeling was observed in the IO cap of Kooy (IOK) (Fig. 1A) as well as in the ventrolateral protrusion (not shown). In more than half of the serially injected rats, Fos labeling was present in the contra-TTX IOC. In one rat, which received two TTX injections, Fos labeling was limited to the contra-TTX subdivisions of the IO and the distribution was the same as reported for a single unilateral TTX injection (see Ref. [53]).

Regardless of the TTX treatment, the temporospatial pattern of Fos expression in the vestibular complex was constant although there was a distinct distributional asym-

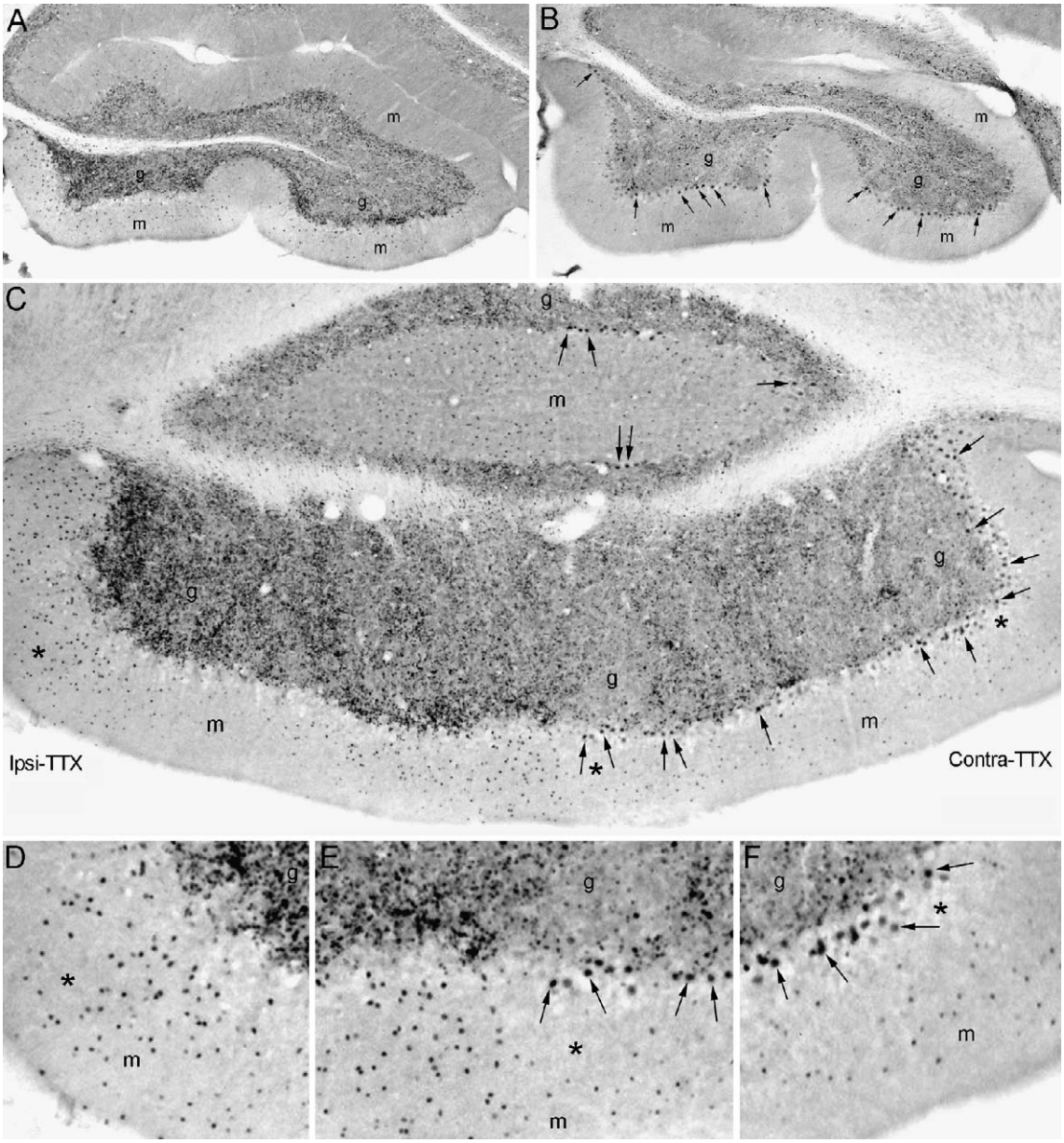

Fig. 4. Digital micrographs of sections through lobule X taken from animals receiving three transtympanic injections of TTX on the same side, 7 days apart and having a final survival time of 2 h. (A) Sagittal section through the ipsi-TTX half of lobule X showing Fos labeling in the granular and molecular cell layers. (B) Sagittal section through the contra-TTX half of lobule X demonstrating Fos expression on PCs (arrows). Note that, relative to the section in A (from the same animal), Fos expression in the molecular (m) and granular (g) layers is substantially less pronounced. (C) Transverse section through the lobule X illustrating the distinct asymmetry in Fos expression in the three layers of cortex. Note that, using the asymmetry in labeling, it is possible to draw an imaginary line that virtually bisects the lobule. Arrows denote the labeled PCs. (D, E, F) Enlargements of regions in C. A corresponding asterisk (*) can be found in C for each asterisk in D–F.

metry between the two sides (Fig. 2A). Of particular note was the dense concentration of intensely labeled nuclei in the dorsomedial aspect of the MVN on the ipsi-TTX side (Fig. 2A). On the contra-TTX side, Fos labeling in the dorsomedial aspect was almost completely absent while labeling elsewhere in the nucleus was prominent relative to the homologous region on the ipsi-TTX side. In addition, sections through more rostral levels of the MVN revealed that there was also a distinct asymmetry (contra-TTX dominance) with regard to Fos labeling in the prepositus hypoglossal and dorsal paragigantocellular nuclei. This temporospatial pattern of Fos labeling was qualitatively identical to that observed following a single unilateral injection of TTX and a survival time of 2 h [53,55].

Fos labeling in lobule X of the cerebellar cortex of TTX treated subjects was elevated bilaterally relative to controls (Figs. 3 and 4). In addition, Fos labeling was distinctly asymmetric in its distribution between the two halves of the lobule. Cells from all three layers of lobule X expressed Fos following serial application of TTX. In the ipsi-TTX half of lobule X many uniformly small, intensely stained nuclei were observed in the granular layer (Fig. 4A and C). Often large numbers of these stained nuclei formed tight clusters and in some instances appeared to form irregular bands through the granular layer (Fig. 4C). There were stained nuclei distributed throughout the molecular layer on the ipsi-TTX side of the lobule (Fig. 4C). By contrast, the contra-TTX half of lobule X displayed a markedly different pattern of Fos labeling. There were far fewer labeled nuclei in the granular layer and only small numbers of faintly stained nuclei scattered in the molecular layer (Fig. 4C,F). The most striking difference pertained to the larger Fos positive nuclei arranged in a linear fashion and localized to the narrow sector between the molecular and granular layers in the zone corresponding to the PC layer (Fig. 4B,C,E and F). The sagittal sections depicted in Fig. 4A and B illustrate that Fos labeling in the three cortical layers extends through the rostrocaudal extent of lobule X. The asymmetry in Fos labeling of lobule X virtually bisects the lobule with high levels of Fos labeling in the granular and molecular layers of the ipsi-TTX half and PC labeling in the contra-TTX half. In the group with the 14-day interval between TTX injections Fos expression in the ipsi-TTX granular and molecular layers was still noticeably elevated relative to the homologous regions in the contra-TTX half of the lobule and in relation to controls (Fig. 3). Further, stained nuclei were still present in the contra-TTX PC layer although most of these profiles were not as intensely stained (Fig. 3E and F) as those observed in subjects with the 7-day regimen.

In one animal, which received two injections of TTX but did not show prominent behavioral effects following the initial injection but did so after the second injection the anatomical results were analogous to that observed following a single TTX injection. In the brainstem Fos labeling in the IO was localized to subdivisions contralateral to TTX treatment, while asymmetric labeling in lobule X consisted of elevated Fos expression in the ipsi-TTX granular and molecular layers but no PC labeling. Further details of Fos labeling following a single unilateral TTX treatment have been published previously [52,53,55].

4. Discussion

The results of the preceding experiments are to the author's knowledge the first published account relating to the CNS response to serial functional deafferentation of the VIIIth cranial nerve. Previous studies have almost exclusively dealt with the process of vestibular compensation (a form of adult plasticity) following permanent ablation (i.e., neurectomy or labyrinthectomy). Recently, behavioral and anatomical results provided support for the use of the transtympanic administration of TTX as a methodology for producing transient functional blockade of the VIIIth nerve in the intact system and that this might be applied in the study of episodic vestibular disruption [55]. In light of the fact that there is now considerable evidence for CNS plasticity in the adult and that it has been shown that unilateral changes in afferent activity in the cochlear component of the VIIIth nerve results in molecular and genetic plasticity at various levels of the auditory system [24,26], it was of interest to investigate the possibility that plastic changes could be elicited in response to episodic vestibular disruption.

In light of the consistent and clearly documented differences in the temporospatial pattern of Fos expression between the two sides of the brainstem and cerebellum, there can be little doubt that these distinct patterns represent differences in the activity in homologous regions in response to episodic vestibular disruption. However, even though the patterns of expression were highly reproducible, the authors cannot rule out the possibility that there could be differences in the numbers of Fos stained profiles between animals.

It was not unexpected that Fos expression would be dramatically altered by episodic vestibular disruption since both ablation [11,14,18,31,37–40] and single treatment TTX [52,53,55] studies have demonstrated asymmetric changes in Fos expression in several brainstem regions. However, it was somewhat unexpected that the observed changes occurred outside of the vestibular complex. While the distribution of induced Fos in the IO and cerebellum following serial TTX treatment was novel the pattern of Fos expression in the central vestibular complex, the termination site for primary vestibular afferents was remarkably similar to that reported following either a single transient block [52,53,55] or following unilateral vestibular ablation [11,31,37–40]. This result not only suggests that cessation of activity in the VIIIth nerve by transient or ablative means initiates a standard asymmetric pattern of activity in the vestibular complex but also that functional blockade promotes a similar pattern of activity at the level of the

vestibular complex regardless of whether it is a unique (i.e., first exposure to TTX blockade) or previously experienced as part of a pattern of deprivation (current experiments). It is worth noting that the observed similarity in the pattern of Fos expression in the vestibular complex following single and multiple TTX treatments is also consistent with the notion that repeated applications of TTX has a similar affect on primary vestibular afferents. Interestingly, in the auditory system, a single unique acoustic stimulus elevates Fos expression and subsequent repetitive exposure to the same stimulus dramatically attenuates Fos expression in the cochlear nuclei [34,35]. Since the present study focused on the temporal and distributional changes in Fos expression, it cannot be ruled out that subtle changes may have occurred in the vestibular complex. However, in light of the temporal change in Fos expression in the IO and dramatic change in Fos expression in the cerebellum, the stable pattern of Fos expression in the vestibular nuclei will in future experiments need to be quantified in order to rule out any significant change in Fos in the vestibular complex. In the auditory system, it has been proposed that the initial elevation of Fos in the cochlear nucleus in response to a novel auditory stimulus and subsequent attenuation of Fos expression following repeat exposure to the same stimulus might serve as an indicator of an underlying process related to auditory learning and hence plasticity [43]. At least within the framework of the current study, there does not appear to be any detectable change in activity at the level of the central vestibular complex paralleling that observed in the auditory system. An explanation for this discrepancy is not immediately apparent but could be related to the type of stimulus (i.e., repetition of a novel sound in the auditory system versus episodic functional deafferentation in the current study) or the number of exposures to a particular stimulus. For instance, three, the largest number of functional blockades used in the current study, may have been insufficient to produce adaptation at the level of the vestibular complex. Alternatively, the possibility must be considered that adaptive changes simply do not take place at the level of the vestibular complex or that any changes occurring cannot be detected under the current protocol.

The temporal change in the pattern of Fos labeling in the ipsilateral IO following episodic blockade was an unexpected finding since previous reports using the TTX model indicate a rigid temporal pattern for Fos expression in the IO [53]. In the current study, serial TTX treatment resulted in bilateral Fos expression in IOA, IOB and IOBe. By contrast, there was no ipsi-TTX Fos expression in the IO following a single unilateral TTX injection except when the survival time was less than 1 h [53]. Studies investigating the induction of Fos following unilateral peripheral vestibular ablation have rarely looked at survival times of less than 2 h and none have reported bilateral Fos expression in the above-mentioned subdivisions of the IO. The protracted expression of Fos in specific IO subdivisions ipsilateral to TTX treatment indicates a modification in the temporal pattern of activity in the IO relative to a single transient event. In light of the particularly robust Fos labeling in the ipsi-TTX IOBe, this subdivision is of particular interest in the present study since it is known receive input from the medial and inferior vestibular nuclei [5,7,28,47] and such inputs could be instrumental in the induction of Fos in specific subdivisions of the IO. Furthermore, efferent projections from the IOBe convey vestibular-related information to the contralateral uvula and nodulus (lobule X) of the cerebellum [5] raising the possibility that these projections might be involved in the induction of Fos in PCs of the contra-TTX lobule X (see PC below). In an elegant double-labeling study, Kitahara et al. [41] showed that the majority (70%) of IOBe neurons expressing Fos following unilateral vestibular ablation projected to the contralateral uvula-nodulus. Furthermore, transsynaptic induction of Fos is now a well-accepted phenomenon [20,23,35,50] and Fos expression has been used to map neuronal activity in a number of pathways including those related to the vestibular system [10,11,13,14,18,19,28–32,37,38,40,41–43,51–53,55].

It is also interesting to note that, although Fos persisted in some ipsi-TTX IO subdivisions following serial TTX treatment, it was noticeably absent in others. For instance, unlike the IOBe which contained persistent ipsi-TTX Fos labeling, the IOK which follows a similar temporal pattern following a single TTX treatment [53] did not contain Fos on the ipsi-TTX side although there was distinct labeling in the contra-TTX IOK. The IOK is known to receive afferent input from the ipsilateral accessory optic system and nucleus of the optic tract and has been shown to be involved in compensatory eye movements [7,47]. Additional visual and eye movement related information might also reach the IOK via a contralateral input from the prepositus hypoglossal nucleus (see Ref. [7]). Since it would appear that the temporospatial pattern of activity in the IOK is equivalent regardless of the type of TTX treatment, it seems reasonable to suggest that information relayed to the IOK is unchanged by episodic functional deafferentation.

The most dramatic change in neuronal activity as monitored by Fos expression was observed in lobule X of the cerebellar cortex. The novel, asymmetric pattern of Fos expression involved neurons located in the granular, molecular and PC layers and was evident with injection intervals of 7 and 14 days but not with the 2-day injection interval. This is in contrast to Fos labeling reported following a single TTX treatment where asymmetric Fos labeling involved only the granular and molecular layers [52]. In a comprehensive vestibular ablation study, Cirelli et al. [11] reported a bilateral but ipsilateral dominant increase in c-*fos* mRNA at 3 h following unilateral labyrinthectomy. In contrast to their mRNA results, they reported no clear induction of Fos protein in the cerebellar cortex. Following unilateral chemical labyrinthectomy, Kaufman et al. [31] reported increased Fos labeling in the ipsi-lesional granular cell layer of both the uvula and nodulus. In a recent study, it

was proposed that the increased Fos expression in the ipsi-TTX granular cell layer of lobule X might be due to mossy fiber inputs originating in the vestibular complex and terminating on granule cells [52].

The explanation for the observed induction of Fos in PCs of lobule X is not readily evident but might be related to the temporal change in Fos expression (i.e., activity) in the ipsi-TTX IO in response to the treatment regime. The asymmetric distribution of Fos positive PCs in the two halves of lobule X appears to square well with the known connectivity of IO inputs to the cerebellum. In addition to being the sole source of climbing fibers and a completely decussated projection, it is generally accepted that olivocerebellar inputs are excitatory and most likely utilize glutamate and/or aspartate as their neurotransmitter(s) [48,59]. Previous reports employing the tremorogenic alkaloid harmaline indicate that, if a sufficient number of IO neurons are activated synchronously at a particular time, there is an equally synchronous activation of PCs [44,45]. O'hearn and Molliver [46] using the related alkaloid, ibogaine, concluded that activation of the inferior olive results in the release of glutamate in a manner that promotes an indirect neurotoxic effect on PCs. Interestingly, at least one known control of transcription factors like Fos is the presynaptic release of neurotransmitter [20–22]. A recent study demonstrated that the systemic administration of harmaline not only induces Fos expression in IO neurons but also transsynaptically in small numbers of PCs [54]. Taken together, the above results seem to support the possibility that protracted activity in neurons of the ipsi-TTX IOBe (efferent projections to lobule X [1,7]) observed following episodic functional deafferentation may be responsible for induction of Fos in PC of the contra-TTX lobule X.

It is now generally accepted that in certain systems transsynaptic stimulation evokes immediate early gene activity with the possibility of subsequent induction of effector genes that are responsible for long-term changes in cell function [35]. It is noteworthy that, in TTX animals with the 7-day injection interval, Fos activity in the ipsi-TTX IO was prominent and so was Fos expression in PCs of the contra-TTX lobule X. On the other hand, in animals with the 14-day injection interval, Fos labeling in the ipsi-TTX IO had largely returned to control levels and PC labeling was attenuated suggesting a further link between Fos expression in the IO and PCs. However, when the injection interval was shortened (i.e., 2 days), Fos labeling was limited to the contra-TTX IO and there was no Fos expression in PCs suggesting that the recovery time between injections might influence Fos activity. In a previous report, it was noted that following a single unilateral TTX injection the time for resolution of behavioral symptoms is greater than 48 h [55] and thus full recovery may be a prerequisite for the observed temporal alteration in Fos expression in the IO and the induction of Fos in PCs. In any event, the provoked induction of Fos in numerous PCs localized to a vestibular related region of the cerebellum as a consequence of serial functional deafferentation strongly implicates these neurons in the response to such perturbations.

Even though induced Fos expression in the contra-TTX IO is robust and known to persist for greater than 2 h following either a single TTX treatment or unilateral vestibular ablation, this does not result in Fos expression in PCs [11,31,53]. In light of this, one might wonder why IO activity in one instance (serial TTX injections) provokes Fos expression in PCs and in others (single TTX treatment or ablation) it does not? Perhaps, the answer to this question lies in the activity of other cerebellar neurons and the type (excitatory or inhibitory) of synaptic inputs they provide to PCs. Based on the known cerebellar circuitry and the knowledge that excitation can drive Fos expression, it is possible to formulate an explanation for the asymmetric pattern of Fos in lobule X. First, with regard to Fos expression in the granule layer, it is important to recognize that, although it has been shown that a large percentage of primary vestibular afferents terminate in the nodulus [8,60], the cessation of activity in the VIIIth nerve due to TTX blockade would most probably eliminate these glutamatergic inputs from contention for a prominent role in the induction of Fos in granule cells. Further, in the present study, there was bilateral elevation of Fos expression in the granule layer of lobule X while primary vestibular afferents terminate unilaterally [8]. Therefore, mossy fiber inputs from sources other than primary afferents are more likely candidates for the induction of Fos in cells of the granular layer. Secondary vestibular inputs to the cerebellum terminate bilaterally (ipsilateral dominant) and might be a possible source of input related to Fos induction in the granule layer of lobule X. Anatomical and histochemical studies have established that a prominent cholinergic input to the nodulus arises from the MVN [4,6,47] and this same mossy fiber input is believed to mediate secondary vestibular information related to postural adjustments [4]. There is also immunocytochemical evidence that some vestibulocerebellar neurons utilize excitatory amino acids like glutamate and/or aspartate as their neurotransmitter(s) [36] although there is a paucity of evidence that these neurons project to lobule X. The prepositus hypoglossi nucleus has a bilateral (ipsilateral dominant) projection to the vermis and neurons in the contra-TTX PrH display intense Fos activity following a single or serial TTX treatment. In light of this mossy fiber inputs from the contra-TTX PrH to the contra-TTX lobule X could contribute to Fos expression in the granular cell layer.

The presumed increase in granule cell activity brought about by mossy fiber input relayed through the vestibular complex and PrH could be the impetus for the observed Fos induction in the molecular layer. In addition to providing inputs to PCs, glutamatergic parallel fibers also synapse on basket and stellate cells and it is conceivable that the marked increase in activity in granule cells observed on the ipsi-TTX side is in part responsible for the Fos induction in cells of the molecular layer. It is worth noting that, in the contra-TTX half of lobule X where Fos expression in the granule layer was less, there was a corresponding decrease in Fos

expression in the molecular layer. With this in mind, one possible explanation for the absence of Fos expression in ipsi-TTX PCs could be the attenuation of excitatory inputs from the contra-TTX IO by the increased activity of inhibitory inputs from ipsi-TTX basket and/or stellate cells. Conversely, the diminished activity in the contra-TTX molecular layer relative to that in the ipsi-TTX half of lobule X might reflect less inhibition of these PCs and thus permitting the protracted activity in the ipsi-TTX IOBe to be an adequate force for induction of Fos in contra-TTX PCs.

The difference between the two halves of lobule X is most intriguing from a functional standpoint although regrettably the current study sheds little light on specific functional aspects. The observation that Fos expression in a particular cell type of lobule X tended to extend through the caudorostral extent of the lobule has implications with respect to the modular concept of cerebellar function. The concept basically states that climbing fibers terminate on PCs in a parasagittal organization, corticonuclear connections are related to olivocerebellar zones, and that this arrangement is at the center of cerebellar function [49,60]. Although clearly delineated parasagittal zones of Fos activity were not identified, Fos expression in sagittal sections does appear to raise the possibility that activity might be modularly arranged. As the singular output element of the cerebellar cortex the role of PCs in the response to episodic vestibular disruption must be considered paramount. The principal targets for PCs are neurons of the deep cerebellar nuclei although Fos labeling in the deep nuclei was not observed in the present study. This might have been expected since increased climbing fiber activity is known to increase firing in PCs, which subsequently produces increased inhibition in neurons of the deep cerebellar nuclei. Interestingly, an earlier study reported Fos induction in the ipsi-TTX medial cerebellar nucleus following a single TTX treatment [52]. However, in the present study, Fos expression was in PCs of the contra-TTX half of lobule X and these cells presumably would inhibit neurons in the medial cerebellar nucleus on the same side (i.e., the contra-TTX side), thus leaving the explanation for the absence of Fos expression in the ipsi-TTX medial cerebellar nucleus following serial blockade unresolved. Following unilateral labyrinthectomy, Cirelli et al. [11] reported a bilateral increase in c-*fos* mRNA with ipsi-lesional dominance in the deep cerebellar nuclei but no corresponding protein expression. Form the standpoint of the current study, it is perhaps more interesting that episodic disruption and ablation share the absence of Fos labeling in the medial cerebellar nucleus while a single blockade of the VIIIth nerve by TTX appears to promote Fos expression in that nucleus. Certainly, methodological differences could account for the absence of Fos expression in the ablation study. Alternatively, differences between the paradigms, ablation versus a single TTX treatment versus episodic vestibular disruption could account for the differences in response. Since it is well established that damage to primary sensory neurons and in particular axotomy can precipitate retrograde cellular changes (see Ref. [2]), it is probable that permanent ablation whether by surgical or other means has consequences that could potentially overshadow and even terminate processes initiated by the acute ablation of vestibular input and that these processes may not mirror those generated by transient or episodic blockade in the intact system. In a related manner, it is also possible that repeat experience with the same aberrant stimulus (i.e., episodic blockade of the VIIIth nerve with TTX) might alter the response relative to a single treatment and subsequently change the temporospatial pattern of Fos expression.

In summary, the results indicate that serial/episodic functional deafferentation of the VIIIth nerve with recovery between each treatment dramatically changes the pattern of activity in the brainstem and cerebellum relative to a single transient event. The prominent stimulus-dependent change in the temporal and spatial pattern of the transcription factor Fos is likely to be indicative of ongoing changes that could presumably modify the chemistry and/or structure of cells. It is important to remember that upregulation of an inducible transcription factor like Fos is not considered a general response to neuronal excitation since many physiologic stimuli do not elicit Fos expression [23,34]. Functionally, the Fos protein can form dimers with other immediate early gene products. Of particular note is its dimerization with c-Jun to form a transcription factor complex called AP-1. AP-1 is capable of triggering the expression of other genes [62], included among these genes is the gene responsible for the growth and plasticity associated protein, GAP-43 [61]. Furthermore, any chemical and/or structural changes in neurons associated with Fos expression could in turn result in a functional change in the response characteristics (e.g., adaptation) of these neurons to a particular stimulus. Whether or not these precise or other events are occurring remains to be elucidated. However, the results do appear to support the notion that unilateral functional deafferentation presented in an episodic paradigm has the potential to precipitate molecular (i.e., slow channel) changes that may lead to transient or long-term changes to neurons integrated into the related circuitry. The fact that differences can be detected following episodic events relative to a single transient event and that Fos expression has been linked to adaptive changes in neurons raises the possibility that, under the right conditions, the vestibular and related systems can be manipulated and the sequence of events leading to adaptive changes observed and evaluated.

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